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<b>(54) Title:</b> CHEMOKINE RECEPTORS 88-2B[CKR-3] AND 88C AND THEIR ANTIBODIES  <b>(57) Abstract</b>  The present invention provides polynucleotides that encode the chemokine receptors 88-2B or 88C and materials and methods for the recombinant production of these two chemokine receptors. Also provided are assays utilizing the polynucleotides which facilitate the identification of ligands and modulators of the chemokine receptors. Receptor fragments, ligands, modulators, and antibodies are useful in the detection and treatment of disease states associated with the chemokine receptors such as atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infection, AIDS, and other inflammatory conditions.		

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**CHEMOKINE RECEPTORS 88-2B [CKR-3] AND 88C AND THEIR ANTIBODIES**

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/661,393 filed June 7, 1996 which was in turn a  
5 continuation-in-part of U.S. Patent Application No. 08/575,967 filed December 20, 1995.

**FIELD OF THE INVENTION**

The present invention relates generally to signal transduction pathways. More particularly, the present invention relates to chemokine  
10 receptors, nucleic acids encoding chemokine receptors, chemokine receptor ligands, modulators of chemokine receptor activity, antibodies recognizing chemokines and chemokine receptors, methods for identifying chemokine receptor ligands and modulators, methods for producing chemokine receptors, and methods for producing antibodies recognizing chemokine receptors.

**BACKGROUND OF THE INVENTION**

Recent advances in molecular biology have led to an appreciation of the central role of signal transduction pathways in biological processes. These pathways comprise a central means by which individual cells in a multicellular organism communicate, thereby coordinating biological  
20 processes. See *Springer, Cell* 76:301-314 (1994), Table I for a model. One branch of signal transduction pathways, defined by the intracellular participation of guanine nucleotide binding proteins (G-proteins), affects a broad range of biological processes.

*Lewin, GENES V* 319-348 (1994) generally discusses G-protein  
25 signal transduction pathways which involve, at a minimum, the following components: an extracellular signal (e.g., neurotransmitters, peptide hormones, organic molecules, light, or odorants), a signal-recognizing receptor (G-protein-coupled receptor, reviewed in *Probst et al., DNA and Cell Biology* 11:1-20 [1992] and also known as GPR or GPCR), and an intracellu-  
30 lar, heterotrimeric GTP-binding protein, or G protein. In particular, these

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pathways have attracted interest because of their role in regulating white blood cell or leukocyte trafficking.

Leukocytes comprise a group of mobile blood cell types including granulocytes (*i.e.*, neutrophils, basophils, and eosinophils), lymphocytes, and monocytes. When mobilized and activated, these cells are primarily involved in the body's defense against foreign matter. This task is complicated by the diversity of normal and pathological processes in which leukocytes participate. For example, leukocytes function in the normal inflammatory response to infection. Leukocytes are also involved in a variety of pathological inflammations. For a summary, see *Schall et al., Curr. Opin. Immunol.* 6:865-873 (1994). Moreover, each of these processes can involve unique contributions, in degree, kind, and duration, from each of the leukocyte cell types.

In studying these immune reactions, researchers initially concentrated on the signals acting upon leukocytes, reasoning that a signal would be required to elicit any form of response. *Murphy, Ann. Rev. Immunol.* 12:593-633 (1994) has reviewed members of an important group of leukocyte signals, the peptide signals. One type of peptide signal comprises the chemokines (*chemoattractant cytokines*), termed intercrines in *Oppenheim et al., Ann. Rev. Immunol.* 9:617-648 (1991). In addition to *Oppenheim et al., Baggiolini et al., Advances in Immunol.* 55:97-179 (1994), documents the growing number of chemokines that have been identified and subjected to genetic and biochemical analyses.

Comparisons of the amino acid sequences of the known chemokines have led to a classification scheme which divides chemokines into two groups: the  $\alpha$  group characterized by a single amino acid separating the first two cysteines (CXC; N-terminus as referent), and the  $\beta$  group, where these cysteines are adjacent (CC). See *Baggiolini et al., supra*. Correlations have been found between the chemokines and the particular leukocyte cell types responding to those signals. *Schall et al., supra*, has reported that the CXC chemokines generally affect neutrophils; the CC chemokines tend to affect monocytes.

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lymphocytes, basophils and eosinophils. For example, *Baggiolini et al., supra*, recited that RANTES, a CC chemokine, functions as a chemoattractant for monocytes, lymphocytes (*i.e.*, memory T cells), basophils, and eosinophils, but not for neutrophils, while inducing the release of histamine  
5 from basophils.

Chemokines were recently shown by Cocchi, *et al.*, *Science*, 270: 1811-1815 (1995) to be suppressors of HIV proliferation. Cocchi, *et al.* demonstrated that RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  suppressed HIV-1, HIV-2 and SIV infection of a CD4<sup>+</sup> cell line designated PM1 and of primary human  
10 peripheral blood mononuclear cells.

Recently, however, attention has turned to the cellular receptors that bind the chemokines, because the extracellular chemokines seem to contact cells indiscriminately, and therefore lack the specificity needed to regulate the individual leukocyte cell types.

15 *Murphy, supra*, reported that the GPCR superfamily of receptors includes the chemokine receptor family. The typical chemokine receptor structure includes an extracellular chemokine-binding domain located near the N-terminus, followed by seven spaced regions of predominantly hydrophobic amino acids capable of forming membrane-spanning  $\alpha$ -helices.  
20 Between each of the  $\alpha$ -helical domains are hydrophilic domains localized, alternately, in the intra- or extra-cellular spaces. These features impart a serpentine conformation to the membrane-embedded chemokine receptor. The third intracellular loop typically interacts with G-proteins. In addition, *Murphy, supra*, noted that the intracellular carboxyl terminus is also capable  
25 of interacting with G-proteins.

The first chemokine receptors to be analyzed by molecular cloning techniques were the two neutrophil receptors for human IL8, a CXC chemokine. *Holmes et al.*, *Science* 253:178-1280 (1991) and *Murphy et al.*, *Science* 253:1280-1283 (1991), reported the cloning of these two receptors for  
30 IL8. *Lee et al.*, *J. Biol. Chem.* 267:16283-16287 (1992), analyzed the cDNAs encoding these receptors and found 77% amino acid identity between the encoded receptors, with each receptor exhibiting features of the G protein

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coupled receptor family. One of these receptors is specific for IL-8, while the other binds and signals in response to IL-8, gro/MGSA, and NAP-2. Genetic manipulation of the genes encoding IL-8 receptors has contributed to our understanding of the biological roles occupied by these receptors. For example, *Cacalano et al., Science 265:682-684 (1994)* reported that deletion of the IL-8 receptor homolog in the mouse resulted in a pleiotropic phenotype involving lymphadenopathy and splenomegaly. In addition, a study of missense mutations described in *Leong et al., J. Biol. Chem. 269:19343-19348 (1994)* revealed amino acids in the IL-8 receptor that were critical for IL-8 binding. Domain swapping experiments discussed in *Murphy, supra*, implicated the amino terminal extracellular domain as a determinant of binding specificity.

Several receptors for CC chemokines have also been identified and cloned. CCCKR1 binds both MIP-1 $\alpha$  and RANTES and causes intracellular calcium ion flux in response to both ligands. *Charo et al., Proc Natl. Acad. Sci. (USA) 91:2752-2756 (1994)* reported that another CC chemokine receptor, MCP-R1 (CCCKR2), is encoded by a single gene that produces two splice variants which differ in their carboxyl terminal domains. This receptor binds and responds to MCP-3 in addition to MCP-1.

A promiscuous receptor that binds both CXC and CC chemokines has also been identified. This receptor was originally identified on red blood cells and *Horuk et al., Science 261:1182-1184 (1993)* reports that it binds IL-8, NAP-2, GRO $\alpha$ , RANTES, and MCP-1. The erythrocyte chemokine receptor shares about 25% identity with other chemokine receptors and may help to regulate circulating levels of chemokines or aid in the presentation of chemokines to their targets. In addition to binding chemokines, the erythrocyte chemokine receptor has also been shown to be the receptor for plasmodium vivax, a major cause of malaria (id.) Another G-protein coupled receptor which is closely related to chemokine receptors, the platelet activating factor receptor, has also been shown to be the receptor for a human pathogen, the bacterium *Streptococcus pneumoniae* (*Cundell et al., Nature 377:435-438 (1995)*).

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In addition to the mammalian chemokine receptors, two viral chemokine receptor homologs have been identified. *Ahuja et al., J. Biol. Chem.* 268:20691-20694 (1993) describes a gene product from *Herpesvirus saimiri* that shares about 30% identity with the IL-8 receptors and binds CXC chemokines. *Neote et al., Cell*, 72:415-425 (1993) reports that human cytomegalovirus contains a gene encoding a receptor sharing about 30% identity with the CC chemokine receptors which binds MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES. These viral receptors may affect the normal role of chemokines and provide a selective pathological advantage for the virus.

Because of the broad diversity of chemokines and their activities, there are numerous receptors for the chemokines. The receptors which have been characterized represent only a fraction of the total complement of chemokine receptors. There thus remains a need in the art for the identification of additional chemokine receptors. The availability of these novel receptors will provide tools for the development of therapeutic modulators of chemokine or chemokine receptor function. It is contemplated by the present invention that such modulators are useful as therapeutics for the treatment of atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infections, and other inflammatory conditions. Alternatively, fragments or variants of the chemokine receptors, or antibodies recognizing those receptors, are contemplated as therapeutics.

### SUMMARY OF THE INVENTION

The present invention provides purified and isolated nucleic acids encoding chemokine receptors involved in leukocyte trafficking. Polynucleotides of the invention (both sense and anti-sense strands thereof) include genomic DNAs, cDNAs, and RNAs, as well as completely or partially synthetic nucleic acids. Preferred polynucleotides of the invention include the DNA encoding the chemokine receptor 88-2B that is set out in SEQ ID NO:3, the DNA encoding the chemokine receptor 88C that is set out in SEQ ID NO:1, and DNAs which hybridize to those DNAs under standard stringent hybridization conditions, or which would hybridize but for the redundancy of

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the genetic code. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM sodium phosphate, pH 6.8 and washing in 0.2X SSC at 55°C. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See *Sambrook et al.*, §§ 9.47-9.51 in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). Also contemplated by the invention are polynucleotides encoding domains of 88-2B or 88C, for example, polynucleotides encoding one or more extracellular domains of either protein or other biologically active fragments thereof. 88-2B extracellular domains correspond to SEQ ID NO:3 and SEQ ID NO:4 at amino acid residues 1-36, 93-107, 171-196, and 263-284. The extracellular domains of 88-2B are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 362-469, 638-682, 872-949, and 1148-1213. Extracellular domains of 88C correspond to SEQ ID NO:1 and SEQ ID NO:2 at amino acid residues 1-32, 89-112, 166-191, and 259-280. The 88C extracellular domains are encoded by polynucleotide sequences that correspond to SEQ ID NO:1 at nucleotides 55-150, 319-390, 550-627, and 829-894. The invention also comprehends polynucleotides encoding intracellular domains of these chemokine receptors. The intracellular domains of 88-2B include amino acids 60-71, 131-151, 219-240, and 306-355 of SEQ ID NO:3 and SEQ ID NO:4. Those domains are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 539-574, 752-814, 1016-1081, and 1277-1426, respectively. The 88C intracellular domains include amino acid residues 56-67, 125-145, 213-235, and 301-352 of SEQ ID NO:1 and SEQ ID NO:2. The intracellular domains of 88C are encoded by polynucleotide sequences corresponding to SEQ ID NO:1 at nucleotides 220-255, 427-489, 691-759, and 955-1110. Peptides corresponding to one or more of the extracellular or intracellular domains, or antibodies raised against those peptides, are contemplated as modulators of receptor activities, especially ligand and G protein binding activities of the receptors.



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The nucleotide sequences of the invention may also be used to design oligonucleotides for use as labeled probes to isolate genomic DNAs encoding 88-2B or 88C under stringent hybridization conditions (*i.e.*, by Southern analyses and Polymerase Chain Reaction methodologies). Moreover, these oligonucleotide probes can be used to detect particular alleles of the genes encoding 88-2B or 88C, facilitating both diagnosis and gene therapy treatments of disease states associated with particular alleles. In addition, these oligonucleotides can be used to alter chemokine receptor genetics to facilitate identification of chemokine receptor modulators. Also, the nucleotide sequences can be used to design antisense genetic elements of use in exploring or altering the genetics and expression of 88-2B or 88C. The invention also comprehends biological replicas (*i.e.*, copies of isolated DNAs made *in vivo* or *in vitro*) and RNA transcripts of DNAs of the invention. Autonomously replicating recombinant constructions such as plasmid, viral, and chromosomal (*e.g.*, YAC) nucleic acid vectors effectively incorporating 88-2B or 88C polynucleotides, and, particularly, vectors wherein DNA effectively encoding 88-2B or 88C is operatively linked to one or more endogenous or heterologous expression control sequences are also provided.

The 88-2B and 88C receptors may be produced naturally, recombinantly or synthetically. Host cells (prokaryotic or eukaryotic) transformed or transfected with polynucleotides of the invention by standard methods may be used to express the 88-2B and 88C chemokine receptors. Beyond the intact 88-2B or 88C gene products, biologically active fragments of 88-2B or 88C, analogs of 88-2B or 88C, and synthetic peptides derived from the amino acid sequences of 88-2B, set out in SEQ ID NO:4, or 88C, set out in SEQ ID NO:2, are contemplated by the invention. Moreover, the 88-2B or 88C gene product, or a biologically active fragment of either gene product, when produced in a eukaryotic cell, may be post-translationally modified (*e.g.*, via disulfide bond formation, glycosylation, phosphorylation, myristoylation, palmitoylation, acetylation, etc.) The invention further contemplates the 88-2B and 88C gene products, or biologically active

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fragments thereof, in monomeric, homomultimeric, or heteromultimeric conformations.

In particular, one aspect of the invention involves antibody products capable of specifically binding to the 88-2B or 88C chemokine  
5 receptors. The antibody products are generated by methods standard in the art using recombinant 88-2B or 88C receptors, synthetic peptides or peptide fragments of 88-2B or 88C receptors, host cells expressing 88-2B or 88C on their surfaces, or 88-2B or 88C receptors purified from natural sources as immunogens. The antibody products may include monoclonal antibodies or  
10 polyclonal antibodies of any source or sub-type. Moreover, monomeric, homomultimeric, and heteromultimeric antibodies, and fragments thereof, are contemplated by the invention. Further, the invention comprehends CDR-grafted antibodies, "humanized" antibodies, and other modified antibody products retaining the ability to specifically bind a chemokine receptor.

The invention also contemplates the use of antibody products  
15 for detection of the 88-2B or 88C gene products, their analogs, or biologically active fragments thereof. For example, antibody products may be used in diagnostic procedures designed to reveal correlations between the expression of 88-2B, or 88C, and various normal or pathological states. In addition,  
20 antibody products can be used to diagnose tissue-specific variations in expression of 88-2B or 88C, their analogs, or biologically active fragments thereof. Antibody products specific for the 88-2B and 88C chemokine receptors may also act as modulators of receptor activities. In another aspect, antibodies to 88-2B or 88C receptors are useful for therapeutic purposes.

25 Assays for ligands capable of interacting with the chemokine receptors of the invention are also provided. These assays may involve direct detection of chemokine receptor activity, for example, by monitoring the binding of a labeled ligand to the receptor. In addition, these assays may be used to indirectly assess ligand interaction with the chemokine receptor. As  
30 used herein the term "ligand" comprises molecules which are agonists and antagonists of 88-2B or 88C, and other molecules which bind to the receptors.

Direct detection of ligand binding to a chemokine receptor may be achieved using the following assay. Test compounds (*i.e.*, putative ligands) are detectably labeled (*e.g.*, radioiodinated). The detectably labeled test compounds are then contacted with membrane preparations containing a chemokine receptor of the invention. Preferably, the membranes are prepared from host cells expressing chemokine receptors of the invention from recombinant vectors. Following an incubation period to facilitate contact between the membrane-embedded chemokine receptors and the detectably labeled test compounds, the membrane material is collected on filters using vacuum filtration. The detectable label associated with the filters is then quantitated. For example, radiolabels are quantitated using liquid scintillation spectrophotometry. Using this technique, ligands binding to chemokine receptors are identified. To confirm the identification of a ligand, a detectably labeled test compound is exposed to a membrane preparation displaying a chemokine receptor in the presence of increasing quantities of the test compound in an unlabeled state. A progressive reduction in the level of filter-associated label as one adds increasing quantities of unlabeled test compound confirms the identification of that ligand.

Agonists are ligands which bind to the receptor and elicit intracellular signal transduction and antagonists are ligands which bind to the receptor but do not elicit intracellular signal transduction. The determination of whether a particular ligand is an agonist or an antagonist can be determined, for example, by assaying G protein-coupled signal transduction pathways. Activation of these pathways can be determined by measuring intracellular  $ca^{++}$  flux, phospholipase C activity or adenylyl cyclase activity, in addition to other assays (see examples 5 and 6).

As discussed in detail in the Examples herein, chemokines that bind to the 88C receptor include RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ , and chemokines that bind to the 88-2B receptor include RANTES.

In another aspect, modulators of the interaction between the 88C and 88-2B receptors and their ligands are specifically contemplated by the invention. Modulators of chemokine receptor function may be identified using

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assays similar to those used for identifying ligands. The membrane preparation displaying a chemokine receptor is exposed to a constant and known quantity of a detectably labeled functional ligand. In addition, the membrane-bound chemokine receptor is also exposed to an increasing quantity of a test compound suspected of modulating the activity of that chemokine receptor. If the levels of filter-associated label correlate with the quantity of test compound, that compound is a modulator of the activity of the chemokine receptor. If the level of filter-associated label increases with increasing quantities of the test compound, an activator has been identified. In contrast, if the level of filter-associated label varies inversely with the quantity of test compound, an inhibitor of chemokine receptor activity has been identified. Testing for modulators of receptor binding in this way allows for the rapid screening of many putative modulators, as pools containing many potential modulators can be tested simultaneously in the same reaction.

The indirect assays for receptor binding involve measurements of the concentration or level of activity of any of the components found in the relevant signal transduction pathway. Chemokine receptor activation often is associated with an intracellular  $\text{Ca}^{++}$  flux. Cells expressing chemokine receptors may be loaded with a calcium-sensitive dye. Upon activation of the expressed receptor, a  $\text{Ca}^{++}$  flux would be rendered spectrophotometrically detectable by the dye. Alternatively, the  $\text{Ca}^{++}$  flux could be detected microscopically. Parallel assays, using either technique, may be performed in the presence and absence of putative ligands. For example, using the microscopic assay for  $\text{Ca}^{++}$  flux, RANTES, a CC chemokine, was identified as a ligand of the 88-2B chemokine receptor. Those skilled in the art will recognize that these assays are also useful for identifying and monitoring the purification of modulators of receptor activity. Receptor activators and inhibitors will activate or inhibit, respectively, the interaction of the receptors with their ligands in these assays.

Alternatively, the association of chemokine receptors with G proteins affords the opportunity of assessing receptor activity by monitoring

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G protein activities. A characteristic activity of G proteins, GTP hydrolysis, may be monitored using, for example,  $^{32}\text{P}$ -labeled GTP.

G proteins also affect a variety of other molecules through their participation in signal transduction pathways. For example, G protein effector molecules include adenylyl cyclase, phospholipase C, ion channels, and phosphodiesterases. Assays focused on any of these effectors may be used to monitor chemokine receptor activity induced by ligand binding in a host cell that is both expressing the chemokine receptor of interest and contacted with an appropriate ligand. For example, one method by which the activity of chemokine receptors may be detected involves measuring phospholipase C activity. In this assay, the production of radiolabeled inositol phosphates by host cells expressing a chemokine receptor in the presence of an agonist is detected. The detection of phospholipase activity may require cotransfection with DNA encoding an exogenous G protein. When cotransfection is required, this assay can be performed by cotransfection of chimeric G protein DNA, for example, Gqi5 (*Conklin, et al., Nature 363:274-276 (1993)*), with 88-2B or 88C DNA and detecting phosphoinositol production when the cotransfected cell is exposed to an agonist of the 88-2B or 88C receptor. Those skilled in the art will recognize that assays focused on G-protein effector molecules are also useful for identifying and monitoring the purification of modulators of receptor activity. Receptor activators and inhibitors will activate or inhibit, respectively, the interaction of the receptors with their ligands in these assays.

Chemokines have been linked to many inflammatory diseases, such as psoriasis, arthritis, pulmonary fibrosis and atherosclerosis. See *Baggiolini et al., supra*. Inhibitors of chemokine action may be useful in treating these conditions. In one example, *Broaddus et al., J. of Immunol. 152:2960-2967 (1994)*, describes an antibody to IL-8 which can inhibit neutrophil recruitment in endotoxin-induced pleurisy, a model of acute inflammation in rabbit lung. It is also contemplated that ligand or modulator binding to, or the activation of, the 88C receptor may be useful in treatment of HIV infection and HIV related disease states. Modulators of chemokine

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binding to specific receptors contemplated by the invention may include antibodies directed toward a chemokine or a receptor, biological or chemical small molecules, or synthetic peptides corresponding to fragments of the chemokine or receptor.

5 Administration of compositions containing 88-2B or 88C modulators to mammalian subjects, for the purpose of monitoring or remediating normal or pathological immune reactions And viral infections including infection by retroviruses such as HIV-1, HIV-2 and SIV is contemplated by the invention. In particular, the invention comprehends the  
10 mitigation of inflammatory responses, abnormal hematopoietic processes, and viral infections by delivery of a pharmaceutically acceptable quantity of 88-2B or 88C chemokine receptor modulators. The invention further comprehends delivery of these active substances in pharmaceutically acceptable compositions comprising carriers, diluents, or medicaments. The invention  
15 also contemplates a variety of administration routes. For example, the active substances may be administered by the following routes: intravenous, subcutaneous, intraperitoneal, intramuscular, oral, anal (*i.e.*, via suppository formulations), or pulmonary (*i.e.*, via inhalers, atomizers, nebulizers, etc.)

In another aspect, the DNA sequence information provided by  
20 the present invention makes possible the development, by homologous recombination or "knockout" strategies [see, e.g. Kapecchi, *Science*, 244:1288-1292 (1989)], of rodents that fail to express a functional 88C or 88-2B chemokine receptor or that express a variant of the receptor. Alternatively, transgenic mice which express a cloned 88-2B or 88C receptor  
25 can be prepared by well known laboratory techniques (Manipulating the Mouse Embryo: A Laboratory Manual, Brigid Hohan, Frank Costantini and Elizabeth Lacy, eds. (1986) Cold Spring Harbor Laboratory ISBN 0-87969-175-I). Such rodents are useful as models for studying the activities of 88C or 88-2B receptors *in vivo*.

30 Other aspects and advantages of the present invention will become apparent to one skilled in the art upon consideration of the following examples.

### **DETAILED DESCRIPTION OF THE INVENTION**

The following examples illustrate the invention. Example 1 describes the isolation of genomic DNAs encoding the 88-2B and 88C chemokine receptors. Example 2 presents the isolation and sequencing of cDNAs encoding human 88-2B and 88C and macaque 88C. Example 3 provides a description of Northern analyses revealing the expression patterns of the 88-2B and 88C receptors in a variety of tissues. Example 4 details the recombinant expression of the 88-2B and 88C receptors. Example 5 describes Ca<sup>++</sup> flux assays, phosphoinositol hydrolysis assays, and binding assays for 88-2B and 88C receptor activity in response to a variety of potential ligands. Experiments describing the role of 88C and 882B as co-receptors for HIV is presented in Examples 6 and 7. The preparation and characterization of monoclonal and polyclonal antibodies immunoreactive with 88C is described in Example 8. Example 9 describes additional assays designed to identify 88-2B or 88C ligands or modulators.

#### **Example 1**

Partial genomic clones encoding the novel chemokine receptor genes of this invention were isolated by PCR based on conserved sequences found in previously identified genes and based on a clustering of these chemokine receptor genes within the human genome. The genomic DNA was amplified by standard PCR methods using degenerate oligonucleotide primers.

Templates for PCR amplifications were members of a commercially available source of recombinant human genomic DNA cloned into Yeast Artificial Chromosomes (*i.e.*, YACs). (Research Genetics, Inc., Huntsville, AL, YAC Library Pools, catalog no. 95011 B). A YAC vector can accommodate inserts of 500-1000 kilobase pairs. Initially, pools of YAC clone DNAs were screened by PCR using primers specific for the gene encoding CCCKR1. In particular, CCCKR(2)-5', the sense strand primer (corresponding to the sense strand of CCCKR1), is presented in SEQ ID NO:15. Primer CCCKR(2)-5' consisted of the sequence 5'-CGTAAGCTTAGAGAAGCCGGGATGGGAA-3', wherein the underlined

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nucleotides are the translation start codon for CCCKR1. The anti-sense strand primer was CCCKR-3' (corresponding to the anti-sense strand of CCCKR1) and its sequence is presented in SEQ ID NO:16. The sequence of CCCKR-3', 5'-GCCTCTAGAGTCAGAGACCAGCAGA-3', contains the reverse complement of the CCCKR1 translation stop codon (underlined). Pools of YAC clone DNAs yielding detectable PCR products (*i.e.*, DNA bands upon gel electrophoresis) identified appropriate sub-pools of YAC clones, based on a proprietary identification scheme. (Research Genetics, Inc., Huntsville, AL). PCR reactions were initiated with an incubation at 94°C for four minutes. Sequence amplifications were achieved using 33 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes.

The sub-pools of YAC clone DNAs were then subjected to a second round of PCR reactions using the conditions, and primers, that were used in the first round of PCR. Results from sub-pool screenings identified individual clones capable of supporting PCR reactions with the CCCKR-specific primers. One clone, 881F10, contained 640 kb of human genomic DNA from chromosome 3p21 including the genes for CCCKR1 and CCCKR2, as determined by PCR and hybridization. An overlapping YAC clone, 941A7, contained 700 kb of human genomic DNA and also contained the genes for CCCKR1 and CCCKR2. Consequently, further mapping studies were undertaken using these two YAC clones. Southern analyses revealed that CCCKR1 and CCCKR2 were located within approximately 100 kb of one another.

The close proximity of the CCCKR1 and CCCKR2 genes suggested that novel related genes might be linked to CCCKR1 and CCCKR2. Using DNA from yeast containing YAC clones 881F10 and 941A7 as templates, PCR reactions were performed to amplify any linked receptor genes. Degenerate oligodeoxyribonucleotides were designed as PCR primers. These oligonucleotides corresponded to regions encoding the second intracellular loop and the sixth transmembrane domain of CC chemokine receptors, as deduced from aligned sequence comparisons of CCCKR1,



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CCCKR2, and V28. V28 was used because it is an orphan receptor that exhibits the characteristics of a chemokine receptor; V28 has also been mapped to human chromosome 3. *Raport et al.*, Gene 163:295-299 (1995). Of further note, the two splice variants of CCCKR2, CCCKR2A and  
5 CCCKR2B, are identical in the second intracellular loop and sixth transmembrane domain regions used in the analysis. The 5' primer, designated V28degf2, contains an internal *Bam*HI site (see below); its sequence is presented in SEQ ID NO:5. The sequence of primer V28degf2 corresponds to DNA encoding the second intracellular loop region of the  
10 canonical receptor structure. See *Probst et al., supra*. The 3' primer, designated V28degr2, contains an internal *Hind*III site (see below); its sequence is presented in SEQ ID NO:6. The sequence of primer V28degr2 corresponds to DNA encoding the sixth transmembrane domain of the canonical receptor structure.

15 Amplified PCR DNA was subsequently digested with *Bam*HI and *Hind*III to generate fragments of approximately 390 bp, consistent with the fragment size predicted from inspection of the canonical sequence. Following endonuclease digestion, these PCR fragments were cloned into pBluescript (Stratagene Inc., LaJolla, CA). A total of 54 cloned fragments  
20 were subjected to automated nucleotide sequence analyses. In addition to sequences from CCCKR1 and CCCKR2, sequences from the two novel chemokine receptor genes of the invention were identified. These two novel chemokine receptor genes were designated 88-2B and 88C.

25 Restriction endonuclease mapping and hybridization were utilized to map the relative positions of genes encoding the receptors 88C, 88-2B, CCCKR1, and CCCKR2. These four genes are closely linked, as the gene for 88C is approximately 18 KBP from the CCCKR2 gene on human chromosome 3p21.

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**Example 2**

Full-length 88-2B and 88C cDNAs were isolated from a macrophage cDNA library by the following procedure. Initially, a cDNA library, described in *Tjoelker et al.*, *Nature* 374:549-553 (1995), was constructed in pRc/CMV (Invitrogen Corp., San Diego, CA) from human macrophage mRNA. The cDNA library was screened for the presence of 88-2B and 88C cDNA clones by PCR using unique primer pairs corresponding to 88-2B or 88C. The PCR protocol involved an initial denaturation at 94°C for four minutes. Polynucleotides were then amplified using 33 cycles of PCR under the following conditions: Denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes. The first primer specific for 88-2B was primer 88-2B-f1, presented in SEQ ID NO:11. It corresponds to the sense strand of SEQ ID NO:3 at nucleotides 844-863. The second PCR primer specific for the gene encoding 88-2B was primer 88-2B-r1, presented in SEQ ID NO:12; the 88-2B-r1 sequence corresponds to the anti-sense strand of SEQ ID NO:3 at nucleotides 1023-1042. Similarly, the sequence of the first primer specific for the gene encoding 88C, primer 88C-f1, is presented in SEQ ID NO:13 and corresponds to the sense strand of SEQ ID NO:1 at nucleotides 453-471. The second primer specific for the gene encoding 88C is primer 88C-r3, presented in SEQ ID NO:14; the sequence of 88C-r3 corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 744-763.

The screening identified clone 777, a cDNA clone of 88-2B. Clone 777 contained a DNA insert of 1915 bp including the full length coding sequence of 88-2B as determined by the following criteria: the clone contained a long open reading frame beginning with an ATG codon, exhibited a Kozak sequence, and had an in-frame stop codon upstream. The DNA and deduced amino acid sequences of the insert of clone 777 are presented in SEQ ID NO:3 and SEQ ID NO:4, respectively. The 88-2B transcript was relatively rare in the macrophage cDNA library. During the library screen, only three 88-2B clones were identified from an estimated total of three million clones.

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Screening for cDNA clones encoding the 88C chemokine receptor identified clones 101 and 134 which appeared to contain the entire 88C coding region, including a putative initiation codon. However, these clones lacked the additional 5' sequence needed to confirm the identity of the initiation codon. The 88C transcript was relatively abundant in the macrophage cDNA Library. During the library screen, it was estimated that 88C was present at one per 3000 transcripts (in a total of approximately three million clones in the library).

RACE PCR (Rapid Amplification of cDNA Ends) was performed to extend existing 88C clone sequences, thereby facilitating the accurate characterization of the 5' end of the 88C cDNA. Human spleen 5'-RACE-ready cDNA was purchased from Clontech Laboratories, Inc., Palo Alto, CA, and used according to the manufacturer's recommendations. The cDNA had been made "5'-RACE-ready" by ligating an anchor sequence to the 5' ends of the cDNA fragments. The anchor sequence is complementary to an anchor primer supplied by Clontech Laboratories, Inc., Palo Alto, CA. The anchor sequence-anchor primer duplex polynucleotide contains an *Eco*RI site. Human spleen cDNA was chosen as template DNA because Northern blots had revealed that 88C was expressed in this tissue. The PCR reactions were initiated by denaturing samples at 94°C for four minutes. Subsequently, sequences were amplified using 35 cycles involving denaturation at 94°C for one minute, annealing at 60°C for 45 seconds, and extension at 72°C for two minutes. The first round of PCR was performed on reaction mixtures containing 2 µl of the 5'-RACE-ready spleen cDNA, 1 µl of the anchor primer, and 1 µl of primer 88c-r4 (100 ng/µl) in a total reaction volume of 50 µl. The 88C-specific primer, primer 88c-r4 (5'-GATAAGCCTCACAG-CCCTGTG-3'), is presented in SEQ ID NO:7. The sequence of primer 88c-r4 corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 745-765. A second round of PCR was performed on reaction mixtures including 1 µl of the first PCR reaction with 1 µl of anchor primer and 1 µl of primer 88C-rlb (100 ng/µl) containing the following sequence

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(5'-GCTAAGCTTGATGACTATCTTTAATGTC-3') and presented in SEQ ID NO:8. The sequence of primer 88C-rlb contains an internal *Hind*III cloning site (underlined). The sequence 3' of the *Hind*III site corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 636-654. The resulting PCR product was digested with *Eco*RI and *Hind*III and fractionated on a 1% agarose gel. The approximately 700 bp fragment was isolated and cloned into pBluescript. Clones with the largest inserts were sequenced. Alternatively, the intact PCR product was ligated into vector pCR using a commercial TA cloning kit (Invitrogen Corp., San Diego, CA) for subsequent nucleotide sequence determinations.

The 88-2B and 88C cDNAs were sequenced using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer Corp., Foster City, CA) and an Applied Biosystems 373A DNA Sequencer. The insert of clone 777 provided the double-stranded template for sequencing reactions used to determine the 88-2B cDNA sequence. The sequence of the entire insert of clone 777 was determined and is presented as the 88-2B cDNA sequence and deduced amino acid sequence in SEQ ID NO:3. The sequence is 1915 bp in length, including 361 bp of 5' untranslated DNA (corresponding to SEQ ID NO:3 at nucleotides 1-361), a coding region of 1065 bp (corresponding to SEQ ID NO:3 at nucleotides 362-1426), and 489 bp of 3' untranslated DNA (corresponding to SEQ ID NO:3 at nucleotides 1427-1915). The 88-2B genomic DNA, described in Example 1 above, corresponds to SEQ ID NO:3 at nucleotides 746-1128. The 88C cDNA sequence, and deduced amino acid sequence, is presented in SEQ ID NO:1. The 88C cDNA sequence is a composite of sequences obtained from RACE-PCR cDNA, clone 134, and clone 101. The RACE-PCR cDNA was used as a sequencing template to determine nucleotides 1-654 in SEQ ID NO:1, including the unique identification of 9 bp of 5' untranslated cDNA sequence in SEQ ID NO:1 at nucleotides 1-9. The sequence obtained from the RACE PCR cDNA confirmed the position of the first methionine codon at nucleotides 55-57 in SEQ ID NO:1, and supported the conclusion that clone 134 and clone 101 contained full-length copies of the 88C coding region. Clone 134 contained

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45 bp of 5' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 10-54), the 1056 bp 88C coding region (corresponding to SEQ ID NO:1 at nucleotides 55-1110), and 492 bp of 3' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 1111-1602). Clone 101 contained 25 bp of 5' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 30-54), the 1056 bp 88C coding region (corresponding to SEQ ID NO:1 at nucleotides 55-1110), and 2273 bp of 3' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 1111-3383). The 88C genomic DNA described in Example 1 above, corresponds to SEQ ID NO:1 at nucleotides 424-809.

The deduced amino acid sequences of 88-2B and 88C revealed hydrophobicity profiles characteristic of GPCRs, including seven hydrophobic domains corresponding to GPCR transmembrane domains. Sequence comparisons with other GPCRs also revealed a degree of identity. Significantly, the deduced amino acid sequences of both 88-2B and 88C had highest identity with the sequences of the chemokine receptors. Table 1 presents the results of these amino acid sequence comparisons.

Table 1

Chemokine Receptors	88-2B	88C
IL-8RA	30%	30%
IL-8RB	31%	30%
CCCKR1	62%	54%
CCCKR2A	46%	66%
CCCKR2B	50%	72%
88-2B	100%	50%
88-C	50%	100%

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Table 1 shows that 88-2B is most similar to CCCKR1 (62 % identical at the amino acid level) and 88C is most similar to CCCKR2 (72 % identical at the amino acid level).

The deduced amino acid sequences of 88-2B and 88C also  
5 reveal the intracellular and extracellular domains characteristic of GPCRs.  
The 88-2B extracellular domains correspond to the amino acid sequence  
provided in SEQ ID NO:3, and SEQ ID NO:4, at amino acid residues 1-36,  
93-107, 171-196, and 263-284. The extracellular domains of 88-2B are  
encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at  
10 nucleotides 362-469, 638-682, 872-949, and 1148-1213. Extracellular  
domains of 88C include amino acid residues 1-32, 89-112, 166-191, and 259-  
280 in SEQ ID NO:1 and SEQ ID NO:2. The 88C extracellular domains are  
encoded by polynucleotide sequences that correspond to SEQ ID NO:1 at  
nucleotides 55-150, 319-390, 550-627, and 829-894. The intracellular  
15 domains of 88-2B include amino acids 60-71, 131-151, 219-240, and 306-355  
of SEQ ID NO:3 and SEQ ID NO:4. Those domains are encoded by  
polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 539-  
574, 752-814, 1016-1081, and 1277-1426, respectively. The 88C intracellular  
domains include amino acid residues 56-67, 125-145, 213-235, and 301-352  
20 of SEQ ID NO:1 and SEQ ID NO:2. The intracellular domains of 88C are  
encoded by polynucleotide sequences corresponding to SEQ ID NO:1 at  
nucleotides 220-255, 427-489, 691-759, and 955-1110.

In addition, a macaque 88C DNA was amplified by PCR from  
macaque genomic DNA using primers corresponding to 5' and 3' flanking  
25 regions of the human 88C cDNA. The 5' primer corresponded to the region  
immediately upstream of and including the initiating Met codon. The 3'  
primer was complementary to the region immediately downstream of the  
termination codon. The primers included restriction sites for cloning into  
expression vectors. The sequence of the 5' primer was  
30 GACAAGCTTCACAGGGTGGAACAAGATG (With the HindIII site underlined)  
(SEQ ID NO: 17) and the sequence of the 3' primer was  
GTCTCTAGACCACTTGAGTCCGTGTCA (with the XbaI site underlined) (SEQ ID

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NO: 18). The conditions of the PCR amplification were 94°C for eight minutes, then 40 cycles of 94°C for one minute, 55°C for forty-five seconds, and 72°C one minute. The amplified products were cloned into the HindIII and XbaI sites of pcDNA3 and a clone was obtained and sequenced. The full length macaque cDNA and deduced amino acid sequences are presented in SEQ ID NOs: 19 and 20, respectively. The nucleotide sequence of macaque 88C is 98% identical to the human 88C sequence. The deduced amino acid sequences are 97% identical.

### Example 3

The mRNA expression patterns of 88-2B and 88C were determined by Northern blot analyses.

Northern blots containing immobilized poly A<sup>+</sup> RNA from a variety of human tissues were purchased from Clontech Laboratories, Inc., Palo Alto, CA. In particular, the following tissues were examined: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes.

A probe specific for 88-2B nucleotide sequences was generated from cDNA clone 478. The cDNA insert in clone 478 contains sequence corresponding to SEQ ID NO: 3 at nucleotides 641-1915. To generate a probe, clone 478 was digested and the insert DNA fragment was isolated following gel electrophoresis. The isolated insert fragment was then radiolabeled with <sup>32</sup>P-labeled nucleotides, using techniques known in the art.

A probe specific for 88C nucleotide sequences was generated by isolating and radiolabeling the insert DNA fragment found in clone 493. The insert fragment from clone 493 contains sequence corresponding to SEQ ID NO: 1 at nucleotides 421-1359. Again, conventional techniques involving <sup>32</sup>P-labeled nucleotides were used to generate the probe.

Northern blots probed with 88-2B revealed an approximately 1.8 kb mRNA in peripheral blood leukocytes. The 88C Northern blots showed an approximately 4 kb mRNA in several human tissues, including a strong signal when probing spleen or thymus tissue and less intense signals when analyzing

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mRNA from peripheral blood leukocytes and small intestine. A relatively weak signal for 88C was detected in lung tissue and in ovarian tissue.

The expression of 88C in human T-cells and in hematopoietic cell lines was also determined by Northern blot analysis. Levels of 88C in CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were very high. The transcript was present at relatively high levels in myeloid cell lines THP1 and HL-60 and also found in the B cell line Jijoye. In addition, the cDNA was a relatively abundant transcript in a human macrophage cDNA library based on PCR amplification of library subfractions.

#### 10 Example 4

The 88-2B and 88C cDNAs were expressed by recombinant methods in mammalian cells.

For transient transfection experiments, 88C was subcloned into the mammalian cell expression vector pBJ1 (Ishi, K. *et. al.*, *J. Biol. Chem* 270:16435-16440 (1995). The construct included sequences encoding a prolactin signal sequence for efficient cell surface expression and a FLAG epitope at the amino terminus of 88C to facilitate detection of the expressed protein. The FLAG epitope consists of the sequence "DYKDDDD." COS-7 cells were transiently transfected with the 88C expression plasmid using Lipofectamine (Life Technology, Inc., Grand Island, NY) following the manufacturer's instructions. Briefly, cells were seeded in 24-well plates at a density of  $4 \times 10^4$  cells per well and grown overnight. The cells were then washed with PBS, and 0.3 mg of DNA mixed with 1.5  $\mu$ l of lipofectamine in 0.25 ml of Opti-MEM was added to each well. After 5 hours at 37°C, the medium was replaced with medium containing 10% FCS. quantitative ELISA confirmed that 88C was expressed at the cell surface in transiently transfected COS-7 cells using the M1 antibody specific for the FLAG epitope (Eastman Co., New Haven, CT).

The FLAG-tagged 88C receptor was also stably transfected into HEK-293 cells, a human embryonic kidney cell line, using transfection reagent DOTAP (N-[1-[(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-



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ammoniummethylsulfate, Boehringer-Mannheim, Inc., Indianapolis, IN) according to the manufacturer's recommendations. Stable lines were selected in the presence of the drug G418. The transfected HEK-293 cells were evaluated for expression of 88C at the cell surface by ELISA, using the M1  
5 antibody to the FLAG epitope. ELISA showed that 88C tagged with the FLAG epitope was expressed at the cell surface of stably transformed HEK-293 Cells.

The 88-2B and 88C cDNAs were used to make stable HEK-293 transfectants. The 88-2B receptor cDNA was cloned behind the cytomegalovirus promoter in pRc/CMV (Invitrogen Corp., San Diego, CA) using a PCR-  
10 based strategy. The template for the PCR reaction was the cDNA insert in clone 777. The PCR primers were 88-2B-3 (containing an internal *Xba*I site) and 88-2B-5 (containing an internal *Hind*III site). The nucleotide sequence of primer 88-2B-3 is presented in SEQ ID NO:9; the nucleotide sequence of  
15 primer 88-2B-5 is presented in SEQ ID NO:10. An 1104 bp region of cDNA was amplified. Following amplification, the DNA was digested with *Xba*I and *Hind*III and cloned into similarly digested pRc/CMV. The resulting plasmid was named 777XP2, which contains 18 bp of 5' untranslated sequence, the entire coding region of 88-2B, and 3 bp of 3' untranslated sequence. For the  
20 88C sequence, the full-length cDNA insert in clone 134 was not further modified before transfecting HEK-293 cells.

To create stably transformed cell lines, the pRc/CMV recombinant clones were transfected using transfection reagent DOTAP (N-[1-  
[(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-  
25 ammoniummethylsulfate, Boehringer-Mannheim, Inc., Indianapolis, IN) according to the manufacturer's recommendations, into HEK-293 cells, a human embryonic kidney cell line. Stable lines were selected in the presence of the drug G418. Standard screening procedures (*i.e.*, Northern blot analyses) were performed to identify stable cell lines expressing the highest  
30 levels of 88-2B and 88C mRNA.

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Example 5A. Ca<sup>++</sup> Flux Assays

To analyze polypeptide expression, a functional assay for chemokine receptor activity was employed. A common feature of signalling through the known chemokine receptors is that signal transduction is associated with the release of intracellular calcium cations. Therefore, intracellular Ca<sup>++</sup> concentration in the transfected HEK-293 cells was assayed to determine whether the 88-2B or 88C receptors responded to any of the known chemokines.

HEK-293 cells, stably transfected with 88-2B, 88C (without the FLAG epitope sequence), or a control coding region (encoding IL8R or CCKR2, see below) as described above, were grown in T75 flasks to approximately 90% confluence in MEM + 10% serum. Cells were then washed, harvested with versene (0.6 mM EDTA, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.14 M NaCl, 3 mM KCl, and 1 mM glucose), and incubated in MEM + 10% serum + 1  $\mu$ M Fura-2 AM (Molecular Probes, Inc., Eugene, OR) for 30 minutes at room temperature. Fura-2 AM is a Ca<sup>++</sup>-sensitive dye. The cells were resuspended in Dulbecco's phosphate-buffered saline containing 0.9 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> (D-PBS) to a concentration of approximately 10<sup>7</sup> cells/ml and changes in fluorescence were monitored using a fluorescence spectrophotometer (Hitachi Model F-4010). Approximately 10<sup>6</sup> cells were suspended in 1.8 ml D-PBS in a cuvette maintained at 37°C. Excitation wavelengths alternated between 340 and 380 nm at 4 second intervals; the emission wavelength was 510 nm. Test compositions were added to the cuvette via an injection port; maximal Ca<sup>++</sup> flux was measured upon the addition of ionomycin.

Positive responses were observed in cells expressing IL-8RA when stimulated with IL-8 and also when CCKR2 was stimulated with MCP-1 or MCP-3. However, HEK-293 cells expressing either 88-2B or 88C failed to show a flux in intracellular Ca<sup>++</sup> concentration when exposed to any of the following chemokines: MCP-1, MCP-2, MCP-3, MIP-1 $\alpha$ , MIP-1 $\beta$ , IL8,

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NAP-2, gro/MGSA, IP-10, ENA-78, or PF-4. (Peprotech, Inc., Rocky Hill, NJ).

Using a more sensitive assay, a  $\text{Ca}^{++}$  flux response to RANTES was observed microscopically in Fura-2 AM-loaded cells expressing 88-2B.

5 The assay involved cells and reagents prepared as described above. RANTES (Regulated on Activation, Normal T Expressed and Secreted) is a CC chemokine that has been identified as a chemoattractant and activator of eosinophils. See Neote *et al.*, *supra*. This chemokine also mediates the release of histamine by basophils and has been shown to function as a

10 chemoattractant for memory T cells *in vitro*. Modulation of 88-2B receptor activities is therefore contemplated to be useful in modulating leukocyte activation.

FLAG tagged 88C receptor was expressed in HEK-293 cells and tested for chemokine interactions in the  $\text{Ca}^{++}$  flux assay. Cell surface

15 expression of 88C was confirmed by ELISA and by FACScan analysis using the M1 antibody. The chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  all induced a  $\text{Ca}^{++}$  flux in 88C-transfected cells when added at a concentration of 100 nM.

$\text{Ca}^{++}$  flux assays can also be designed to identify modulators of

20 chemokine receptor binding. The preceding fluorimetric or microscopic assays are carried out in the presence of test compounds. If  $\text{Ca}^{++}$  flux is increased in the presence of a test compound, that compound is an activator of chemokine receptor binding. In contrast, a diminished  $\text{Ca}^{++}$  flux identifies the test compound as an inhibitor of chemokine receptor binding.

25 B. Phosphoinositol Hydrolysis

Another assay for ligands or modulators involves monitoring phospholipase C activity, as described in Hung *et al.*, *J. Biol. Chem.* 116:827-832 (1992). Initially, host cells expressing a chemokine receptor are loaded with  $^3\text{H}$ -inositol for 24 hours. Test compounds (*i.e.*, potential ligands) are

30 then added to the cells and incubated at 37°C for 15 minutes. The cells are then exposed to 20 mM formic acid to solubilize and extract hydrolyzed

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metabolites of phosphoinositol metabolism (*i.e.*, the products of phospholipase C-mediated hydrolysis). The extract is subjected to anion exchange chromatography using an AG1X8 anion exchange column (formate form). Inositol phosphates are eluted with 2 M ammonium formate/0.1 M formic acid and the  $^3\text{H}$  associated with the compounds is determined using liquid scintillation spectrophotometry. The phospholipase C assay can also be exploited to identify modulators of chemokine receptor activity. The aforementioned assay is performed as described, but with the addition of a potential modulator. Elevated levels of detectable label would indicate the modulator is an activator; depressed levels of the label would indicate the modulator is an inhibitor of chemokine receptor activity.

The phospholipase C assay was performed to identify chemokine ligands of the FLAG-tagged 88C receptor. Approximately 24 hours after transfection, COS-7 cells expressing 88C were labeled for 20-24 hours with *myo*-[2- $^3\text{H}$ ]inositol (1  $\mu\text{Ci/ml}$ ) in inositol-free medium containing 10% dialyzed FCS. Labeled cells were washed with inositol-free DMEM containing 10 mM LiCl and incubated at 37°C for 1 hour with inositol-free DMEM containing 10 mM LiCl and one of the following chemokines: RANTES, MIP-1 $\beta$ , MIP-1 $\alpha$ , MCP-1, IL-8, or the murine MCP-1 homolog JE. Inositol phosphate (IP) formation was assayed as described in the previous paragraph. After incubation with chemokines, the medium was aspirated and cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid (30 min). Supernatant fractions were loaded onto AG1-X8 Dowex columns (Biorad, Hercules, CA), followed by immediate addition of 3 ml of 50 mM  $\text{NH}_4\text{OH}$ . The columns were then washed with 4 ml of 40 mM ammonium formate, followed by elution with 2 M ammonium formate. Total inositol phosphates were quantitated by counting beta-emissions.

Because it has been shown that some chemokine receptors, such as IL8RA AND IL8RB, require cotransfection with an exogenous G protein before signalling can be detected in COS-7 cells, the 88C receptor was co-expressed with the chimeric G protein Gqi5 (*Conklin, et al., Nature* 363:274-276, (1993)). Gqi5 is a G protein which has the carboxyl terminal five amino

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acids of Gi (which bind to the receptor) spliced onto G $\alpha$ q. Co-transfection with Gqi5 significantly potentiates signaling by CCCKR1 and CCKR2B. Co-transfection with Gqi5 revealed that 88C signaled well in response to RANTES, MIP-1 $\beta$ , and MIP-1 $\alpha$ , but not in response to MCP-1, IL-8 or the murine MCP-1 homologue JE. Dose-response curves revealed EC<sub>50</sub> values of 1nM for RANTES, 6nM for MIP-1 $\beta$ , and 22nM for MIP-1 $\alpha$ .

88C is the first cloned human receptor with a signaling response to MIP-1 $\beta$ . Compared with other CC chemokines, MIP-1 $\beta$  clearly has a unique cellular activation pattern. It appears to activate T cells but not monocytes (Baggiolini et al., Supra) which is consistent with receptor stimulation studies. For example, while MIP-1 $\beta$  binds to CCCKR1, it does not induce calcium flux (Neote et al., Supra). In contrast, MIP-1 $\alpha$  and RANTES bind to and causes signalling in CCCKR1 and CCCKR5 (RANTES also causes activation of CCCKR3). MIP-1 $\beta$  thus appears to be much more selective than other chemokines of the CC chemokine family. Such selectivity is of therapeutic significance because a specific beneficial activity can be stimulated (such as suppression of HIV infection) without stimulating multiple leukocyte populations which results in general pro-inflammatory activities.

### C. BINDING ASSAYS

Another assay for receptor interaction with chemokines was a modification of the binding assay described by Ernst *et al. J. Immunol.* 152:3541-3549 (1994). MIP-1 $\beta$  as labeled using the Bolton and Hunter reagent (di-iodide, NEN, Wilmington, DE), according to the manufacturer's instructions. Unconjugated iodide was separated from labeled protein by elution using a PD-10 column (Pharmacia) equilibrated with PBS and BSA (1% w/v). The specific activity was typically 2200 Ci/mmol. Equilibrium binding was performed by adding <sup>125</sup>I-labeled ligand with or without a 100-fold excess of unlabeled ligand, to 5 X 10<sup>5</sup> HEK-293 cells transfected with 88C tagged with the FLAG epitope in polypropylene tubes in a total volume of 300  $\mu$ l (50 mM HEPES pH 7.4, 1 mM CaCl<sub>2</sub>, MgCl<sub>2</sub>, 0.5% BSA) and incubating for 90 minutes at 27°C with shaking at 150 rpm. The cells were

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collected, using a Skatron cell harvester (Skatron Instruments Inc., Sterling, VA), on glass fiber filters presoaked in 0.3% polyethyleneimine and 0.2% BSA. After washing, the filters were removed and bound ligand was quantitated by counting gamma emissions. Ligand binding by competition with unlabeled ligand was determined by incubation of  $5 \times 10^5$  transfected cells (as above) with 1.5 nM of radiolabeled ligand and the indicated concentrations of unlabeled ligand. The samples were collected, washed and counted as above. The data was analyzed using the curve-fitting program Prism (GraphPad Inc., San Diego, CA) and the iterative non-linear regression program, LIGAND (PM220).

In equilibrium binding assays, 88C receptor bound radiolabeled MIP-1 $\beta$  in a specific and saturable manner. Analysis of this binding data by the method of Scatchard revealed a dissociation constant ( $K_d$ ) of 1.6 nM. Competition binding assays using labeled MIP-1 $\beta$  revealed high-affinity binding of MIP-1 $\beta$  ( $IC_{50} = 7.4$  nM), RANTES ( $IC_{50} = 6.9$  nM), and MIP-1 $\alpha$  ( $IC_{50} = 7.4$  nM), consistent with the signaling data obtained in transiently transfected COS-7 cells as discussed in section B above.

#### Example 6

The chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES have been shown to inhibit replication of HIV-1 and HIV-2 in human peripheral blood mononuclear cells and PM1 cells (*Cocchi, et. al., supra*). In view of this finding and in view of the results described in Example 5, the present invention contemplates that activation of or ligand binding to the 88C receptor may provide a protective role in HIV infection.

Recently, it has been reported that the orphan G protein-coupled receptor, fusin, can act as a co-receptor for HIV entry. Fusin/CXCR4 in combination with CD4, the primary HIV receptor, apparently facilitates HIV infection of cultured T cells (*Feng, et al., Science 272:872-877 (1996)*). Based upon the homology of fusin to chemokine receptors and the chemokine binding profile of 88C, and because 88C is constitutively expressed in T cells

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and abundantly expressed in macrophages, 88C is likely to be involved in viral and HIV infection.

The function of 88C and 88-2B as co-receptors for HIV was determined by transfecting cells which express CD4 with 88C or 88-2B and challenging the co-transfected cells with HIV. Only cells expressing both CD4 and a functional co-receptor for HIV become infected. HIV infection can be determined by several methods. ELISAs which test for expression of HIV antigens are commercially available, for example Coulter HIV-1 p24 antigen assay (US Patent Nos. 4,886,742), Coulter Corp., 11800 SW 147th Ave., Miami, FL 33196. Alternatively, the test cells can be engineered to express a reporter gene such as *LACZ* attached to the HIV LTR promoter [Kimpton *et al.*, *J. Virol.* 66:2232-2239 (1992)]. In this method, cells that are infected with HIV are detected by a colorimetric assay.

88C was transiently transfected into a cat cell line, CCC [Clapham, *et al.*, 181:703-715 (1991)], which had been stably transformed to express human CD4 (CCC-CD4). These cells are normally resistant to infection by any strain of HIV-1 because they do not endogenously express 88C. In these experiments, CCC/CD4 cells were transiently transfected with 88C cloned into the expression vector pcDNA3.1 (Invitrogen Corp., San Diego, CA) using lipofectamine (Gibco BRL, Gaithersburg, MD). Two days after transfection, cells were challenged with HIV. After 4 days of incubation, cells were fixed and stained for p24 antigen as a measure of HIV infection. 88C expression by these cells rendered them susceptible to infection by several strains of HIV-1. These strains included four primary non-syncytium-inducing HIV-1 isolates (M23, E80, SL-2 and SF-162) which were shown to use only 88C as a co-receptor but not fusin. Several primary syncytium-inducing strains of HIV-1 (2006, M13, 2028 and 2076) used either 88C or fusin as a co-receptor. Also, two established clonal HIV-1 viruses (GUN-1 and 89.6) used either 88C or fusin as a co-receptor.

It has been reported that some strains of HIV-2 can infect certain CD4-negative cell lines, thus implying a direct interaction of HIV-2 with a receptor other than CD4 [Clapham, *et al.*, *J. Virol.* 66:3531-3537

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(1992)] For some strains of HIV-2, this infection is facilitated by the presence of soluble CD4 (sCD4). Since 88-2B shares high sequence similarity with other chemokine receptors that act as HIV co-receptors (namely 88C and fusin), 88-2B was considered to be a likely HIV-2 co-receptor. The role of 88-2B as an HIV-2 co-receptor was demonstrated using HIV-2 strain ROD/B. Cat CCC cells which do not endogeneously express CD4 were transfected with 88-2B. In these experiments, cells were transfected with pcDNA3.1 containing 88-2B using lipofectamine and infected with HIV-2 48 hours later. Three days after infection, cells were immunostained for the presence of HIV-2 envelope glycoproteins. The presence of sCD4 during HIV-2<sub>ROD/B</sub> challenge increased the infection of these cells by 10-fold. The entry of HIV-2 into the 88-2B transfected cells could be blocked by the presence of 400-800 ng/ml eotaxin, one of the ligands for 88-2B. The baseline infectivity levels of CCC/88-2B (with no soluble CD4) were equivalent to CCC cells which were not transfected with 88-2B.

The role of 88-2B and 88C as co-receptors for HIV was confirmed by preparing and challenging cell lines stably transformed to express 88C or 88-2B with various strains of HIV and SIV. These results are described in Example 7.

Alternatively, the co-receptor role of 88C and 88-2B can be demonstrated by an experimental method which does not require the use of live virus. In this method, cell lines co-expressing 88C or 88-2B, CD4 and a *LACZ* reporter gene are mixed with a cell line co-expressing the HIV envelope glycoprotein (ENV) and a transcription factor for the reporter gene construct (Nussbaum, *et al.*, 1994 *J. Virol.* 68:5411). Cells expressing a functional co-receptor for HIV will fuse with the ENV expressing cells and thereby allow expression of the reporter gene. In this method, detection of reporter gene product by colorimetric assay indicates that 88C or 88-2B function as a co-receptor for HIV.

The mechanism by which chemokines inhibit viral infection has not yet been elucidated. One possible mechanism involves activation of the receptor by binding of a chemokine. The binding of the chemokine leads to



signal transduction events in the cell that renders the cell resistant to viral infection and/or prevents replication of the virus in the cell. Similar to interferon induction, the cell may differentiate such that it is resistant to viral infection, or an antiviral state is established. Alternatively, a second  
5 mechanism involves direct interference with viral entry into cells by blocking access of viral envelope glycoproteins to the co-receptor by chemokine binding. In this mechanism, G-protein signalling is not required for chemokine suppression of HIV infection.

To distinguish between two mechanisms by which 88C or 88-2B  
10 may function as co-receptors for viral or HIV infection, chemokine binding to the receptor is uncoupled from signal transduction and the effect of the chemokine on suppression of viral infection is determined.

Ligand binding can be uncoupled from signal transduction by the addition of compounds which inhibit G-protein mediated signaling. These  
15 compounds include, for example, pertussis toxin and cholera toxin. In addition, downstream effector polypeptides can be inhibited by other compounds such as wortmannin. If G-protein signalling is involved in suppression of viral infection, the addition of such compounds would prevent suppression of viral infection by the chemokine. Alternatively, key residues  
20 or receptor domains of 88C or 88-2B receptor required for G-protein coupling can be altered or deleted such that G-protein coupling is altered or destroyed but chemokine binding is not affected.

Under these conditions, if chemokines are unable to suppress viral or HIV infection, then signaling through a G-protein is required for  
25 suppression of viral or HIV infection. If however, chemokines are able to suppress viral infection, then G-protein signaling is not required for chemokine suppression of viral infection and the protective effects of chemokines may be due to the chemokine blocking the availability of the receptor for the virus.

30 Another approach involves the use of antibodies directed against 88C or 88-2B. Antibodies which bind to 88C or 88-2B which can be shown not to elicit G-protein signaling may block access to the chemokine or viral

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binding site of the receptor. If in the presence of antibodies to 88C or 88-2B, viral infection is suppressed, then the mechanism of the protective effects of chemokines is blocking viral access to its receptor. Feng, *et al.* Reported that antibodies to the amino terminus of the fusin receptor suppressed HIV  
5 infection (Feng, *et al.*, 1996).

### Example 7

Cell lines were stably transformed with 88C or 88-2B to further delineate the role of 88C and 88-2B in HIV infection. Kimpton and Emerman, "Detection of Replication-Competent and Pseudotyped Human  
10 Immunodeficiency Virus with a Sensitive Cell Line on the Basis of Activation of an Integrated Beta-Galactosidase Gene," *J. Virol.*, 66(5):3026-3031 (1992) previously described an indicator cell line, herein identified as HeLa-MAGI cells. HeLa-MAGI cells are HeLa cells that have been stably transformed to express CD4 as well as integrated HIV-1 LTR which drives expression of a  
15 nuclear localized  $\beta$ -galactosidase gene. Integration of an HIV provirus in the cells leads to production of the viral transactivator, Tat, which then turns on expression of the  $\beta$ -galactosidase gene. The number of cells that stain positive with X-gal for  $\beta$ -galactosidase activity *in situ* is directly proportional to the number of infected cells.

20 These HeLa-MAGI cells can detect lab-adapted isolates of HIV-1 but only a minority of primary isolates [Kimpton and Emerman, *supra*], and cannot detect most SIV isolates [Chackerian *et al.*, "Characterization of a CD4-Expressing Macaque Cell Line that can Detect Virus After A Single Replication Cycle and can be infected by Diverse Simian Immunodeficiency  
25 Virus Isolates." *Virology*, 213(2):6499-6505 (1995)].

In addition, Harrington and Geballe, "Co-Factor Requirement for Human Immunodeficiency Virus Type 1 Entry into a CD4-Expressing Human Cell Line, *J. Virol.*, 67:5939-5947 (1993) described a cell line based on U373 cells that had been engineered to express CD4 and the same LTR- $\beta$ -  
30 galactosidase construct. It was previously shown that this cell line, herein identified as U373-MAGI, could not be infected with any HIV (M or T-tropic)

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strain of HIV, but could be rendered susceptible to infection by fusion with HeLa cells [Harrington and Geballe, *supra*].

In order to construct indicator cell lines that could detect either Macrophage or T cell tropic viruses, epitope-tagged 88C or 88-2B encoding DNA was transfected into HeLa-MAGI or U373-MAGI cells by infection with a retroviral vector to generate HeLa-MAGI-88C or U373-MAGI-88C cell lines, respectively. Expression of the co-receptors on the cell surface was demonstrated by immunostaining live cells using the anti-FLAG M1 antibody and by RT-PCR.

The 88C and 88-2B genes utilized to construct HeLa-MAGI-88C and U373-MAGI-88C included sequences encoding the prolactin signal peptide followed by a FLAG epitope as described in Example 4. This gene was inserted into the retroviral vector pBabe-Puro [Morgenstern and Land *Nucleic Acids Research*, 18(12):3587-3596 (1990)]. High titer retroviral vector stocks pseudotyped with the VSV-G protein were made by transient transfection as described in Bartx *et al.*, *J. Virol.* 70:2324-2331 (1996), and used to infect HeLa-MAGI and U373-MAGI cells. Cells resistant to 0.6  $\mu$ g/ml puromycin (HeLa) or 1  $\mu$ g/ml puromycin (U373) were pooled. Each pool contained at least 1000 independent transduction events. An early passage (passage 2) stock of the original HeLa-MAGI cells [Kimpton and Emerman, *supra*] was used to create HeLa-MAGI-88C cells.

Infections of the indicator cell lines with HIV were performed in 12-well plates with 10-fold serial dilutions of 300  $\mu$ l of virus in the presence of 30  $\mu$ g/ml DEAE-Dextran as described [Kimpton and Emerman, *supra*].

All HIV-1 strains and SIV<sub>mac239</sub> were all obtained from the NIH AIDS Reference and Reagent Program. Molecular clones of primary HIV-2<sub>7312A</sub> [Gao *et al.*, "Genetic Diversity of Human Immunodeficiency Virus Type 2: Evidence for Distinct Sequence Subtypes with Differences in Virus Biology," *J. Virol.*, 68(11):7433-7447 (1992)] and SIVsmPbJ1.9 [Dewhurst *et al.*, "Sequence Analysis and Acute Pathogenicity of Molecularly Cloned SIV<sub>smun</sub>-PBj14," *Nature*, 345:636-640 (1990)] were obtained from B. Hahn

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(UAB). All other SIV<sub>mac</sub> isolates were obtained from Julie Overbaugh (U. Washington, Seattle). Stocks from cloned proviruses were made by transient transfection of 293 cells. Other viral stocks were made by passage of virus in human peripheral blood mononuclear cells or in CEMx174 cells (for SIV stocks.) Viral stocks were normalized by ELISA or p24<sup>gag</sup> (Coulter Immunology) or p27<sup>gag</sup> (Coulter Immunology) for HIV-1 and HIV-2/SIV, respectively, using standards provided by the manufacturer.

U373-MAGI-88C cells and U373-MAGI cells (controls) and were infected with limiting dilutions of a T-tropic strain of HIV-1 (HIV<sub>LAI</sub>), an M-tropic strain (HIV<sub>YU-2</sub>), and an SIV isolate, SIV<sub>MAC</sub>239. Infectivity was measured by counting the number of blue cells per well per volume of virus (Table 2).

Table 2

<u>virus strain</u> <sup>a</sup>	<u>titer on cell line (IU/ml)</u> <sup>b</sup>	
	U373-MAGI	U373-MAGI-88C
HIV-1 <sub>LAI</sub>	< 100	< 100
HIV-1 <sub>YU-2</sub>	< 100	2.2 x 10 <sup>6</sup>
SIV <sub>MAC</sub> 239	1.2 x 10 <sup>3</sup>	4 x 10 <sup>5</sup>

<sup>a</sup> Viruses derived by transfection of molecular clones into 293 cells.

<sup>b</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume.

Two days after infection, cells were fixed and stained for  $\beta$ -galactosidase activity with X-gal. The U373-derived MAGI cells were stained for 120 minutes at 37°C and the HeLa-derived MAGI cells were stained for 50 minutes at 37°C. Background staining of non-infected cells never exceeded more than approximately three blue cells per well. Only dark blue cells were counted, and syncytium with multiple nuclei were counted as a single infected cell. The infectious titer is the number of blue cells per well multiplied by the dilution of virus and normalized to 1 ml. The titer of

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HIV<sub>YU.2</sub> on U373-MAGI-88C cells was  $2 \times 10^6$ . In contrast, the titer of HIV-  
1<sub>LAI</sub> was less than 100 on U373-MAGI-88C. Thus, the specificity of a  
particular HIV strain for 88C varied by four orders of magnitude.

Although SIV<sub>MAC</sub>239 infection was increased to  $4 \times 10^5$  in  
5 U373-MAGI-88C it also clearly infected U373-MAGI cells (Table 2).

Next, a series of primary uncloned HIV strains and cloned M-  
tropic strains of HIV-1 were analyzed for their ability to infect indicator cell  
lines that express 88C.

As described above, HeLa-MAGI and HeLa-MAGI-88C cells  
10 were infected with limiting dilutions of various HIV strains. The two cloned  
M-tropic viruses, HIV<sub>JR-CSF</sub> and HIV<sub>YU.2</sub>, both infected HeLa-MAGI-88C, but  
not HeLa-MAGI cells, showing that both strains use 88C as a co-receptor  
(Table 3. See note c). However, a great disparity in the ability of each of  
these two viral strains to infect HeLa-MAGI-88C cells was observed.  $6.2 \times$   
15  $10^5$  IU/ml for HIV<sub>YU.2</sub> and  $1.2 \times 10^4$  for HIV<sub>JR-CSF</sub>. The infectivity of virus  
stock (Table 3) is the number of infectious units per physical particle  
(represented here by the amount of viral core protein). In addition, it was  
observed that the infectivity of these two cloned viral strains differed by over  
50-fold in viral stocks that were independently prepared.

20 The variability of infectivity of primary viral isolates was  
further examined by analyzing a collection of twelve different uncloned virus  
stocks from three different clades (Table 3). Three clade A primary isolates,  
three clade E isolates, and three additional clade B isolates from  
geographically diverse origins were used. With all nine strains, the primary  
25 strains of HIV could be detected on HeLa-MAGI-88C cells, but not on HeLa-  
MAGI cells (Table 3). However, the efficacy of infection varied from five  
infectious units per ng p24<sup>gag</sup> to over 100 infectious units per ng p24<sup>gag</sup> (table  
3). These results indicate that absolute infectivity of M-tropic strains varies  
considerably and is independent of clade. A hypothesis that may explain this  
30 discrepancy may involve the affinity of the V3 loop of each viral strain for  
88C after CD4 binding [Trkola *et al.*, *Nature*, 384(6605):184-187 (1996); Wu  
*et al.*, *Nature*, 384(6605):179-183 (1996)].

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Table 3

<u>virus strain<sup>a</sup></u>	<u>viral sub-type</u> <u>(country of origin)<sup>b</sup></u>	<u>titer (IU/ml) on</u> <u>HeLa-MAGI-88C<sup>c</sup></u>	<u>P24<sup>gag</sup> ng/ml</u>	<u>Infectivity<sup>d</sup></u>
HIV-1 <sub>YU-2</sub>	B (USA)	$6.2 \times 10^5$	2200	281
HIV-1 <sub>JR-CSF</sub>	B (USA)	12000	2800	4.2
5 HIV-1 <sub>TH020</sub>	E (Thailand)	4133	93	44
HIV-1 <sub>TH021</sub>	E (Thailand)	4967	52	96
HIV-1 <sub>TH022</sub>	E (Thailand)	200	15	13
HIV-1 <sub>US660</sub>	B (USA)	2367	127	19
HIV-1 <sub>UG031</sub>	A (Uganda)	1633	71	23
10 HIV-1 <sub>RW009</sub>	A (Rwanda)	3333	158	21
HIV-1 <sub>RW026</sub>	A (Rwanda)	739	143	5.2
HIV-1 <sub>US727</sub>	B (USA)	14,067	289	49
HIV-1 <sub>US056</sub>	B (USA)	5833	284	21
HIV-1 <sub>LAI</sub>	B (France)	$2.8 \times 10^5$	167	1600

15 <sup>a</sup> HIV-1<sub>YU-2</sub> and HIV-1<sub>JR-CSF</sub> were derived by transfection of molecular clones. All others were tested as crude supernatants of uncloned viral stocks derived from infection of heterologous peripheral blood mononuclear cells.

20 <sup>b</sup> Clade designation according to Myers *et al.*, 1995 for the env gene; country of origin refers to the country of residence of the HIV-positive individual from whom blood was obtained for viral isolation (World Health Organization Viral Isolate Program).

25 <sup>c</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume. All viruses, except HIV-1<sub>LAI</sub>, had less than 10 IU/ml when tested on HeLa-MAGI cells without 88C. HIV<sub>LAI</sub>, a T-tropic strain, has a titer of  $2.8 \times 10^5$  on HeLa-MAGI cells with or without 88C.

30 <sup>d</sup> Infectivity is the infectious units per ng P24<sup>gag</sup> (column four divided by column five).

The ability of the HeLa-MAGI-88C cells to detect HIV-2 and other SIV strains was also determined. HIV-2<sub>Rod</sub> has been reported to use fusin as a receptor even in the absence of CD4 [Endres *et al.*, *Cell*,

87(4):745-756 (1996)]. HIV-2<sub>Rod</sub> is able to infect HeLa-MAGI cells, however its infectivity is enhanced at least 10-fold in HeLa-MagI-88C (Table 4). HeLa cells endogenously express fusin. Thus, the molecular clone of HIV-2<sub>Rod</sub> is dual tropic, and is able to use 88C as one of its co-receptors in addition to CXCR4. Similarly, a primary strain of HIV-2<sub>7312A</sub> infected HeLa-MAGI-88C cells and not the HeLa-MAGI cells, indicating that like primary strain of HIV-1, it uses 88C as a receptor.

Table 4

	virus strain <sup>a</sup>	reference	titer (IU/ml) on HeLa-MAGI <sup>b</sup>	titer (IU/ml) on HeLa-MAGI-88C	Infectivity on HeLa-MAGI-88C <sup>c</sup>
10	HIV-2 <sub>Rod</sub>	(Guyader <i>et al.</i> , 1987)	967	5900	13
	HIV-2 <sub>7312A</sub>	(Gao <i>et al.</i> , 1994)	<30	6500	17
	SIV <sub>MAC</sub> 239	(Naidu <i>et al.</i> , 1988)	<30	20900	90
	SIV <sub>MNEC</sub> 18	(Overbaugh <i>et al.</i> , 1991)	<30	15700	19
	SIV <sub>MNE</sub> 170	(Rudensey <i>et al.</i> , 1995)	<30	10700	27
15	SIV <sub>SM</sub> Pbj1.9	(Dewhurst <i>et al.</i> , 1990)	<30	776	ND <sup>d</sup>
	SIV <sub>AGM</sub> 9063	(Hirsch <i>et al.</i> , 1995)	<30	50	<1

<sup>a</sup> HIV-2 stocks. SIV<sub>SM</sub>Pbj1.9, and SIV<sub>AGM</sub>9063 were tested directly after by transfection of molecular clones in 293 cells. All others were derived from transfection of molecular clones and subsequently amplified in CEMx174 cells.

<sup>b</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume. All viruses in this panel were also negative on HeLa-MAGI-88-2B.

<sup>c</sup> Infectivity is the infectious units (on 88C expressing cells) per ng P27<sup>ELISA</sup> determined by ELISA.

<sup>d</sup> ND, not determined.

None of the SIV strains tested infected the HeLa-MAGI cells (Table 4), and none infected HeLa-MAGI cells that expresses 88-2B. This indicates that an alternative co-receptor used by SIV in U373 cells is not expressed in HeLa cells, and is not 88-2B. All SIV strains tested infected the

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HeLa-MAGI-88C cells to some extent (Table 3) indicating that all of the tested SIV strains use at least 88C as one of their co-receptors.

The classification of M-tropic and T-tropic strains of HIV in the past has often been correlated with another designation "non-syncytium inducing" (NSI), and "syncytium inducing" (SI), respectively. Assays based on the cell lines described herein are sensitive to syncytium formation. The infected cells can form large and small foci of infection containing multiple nuclei [Kimpton and Emerman, *supra*].

Experiments using multiple different viral strains and U373-MAGI-88C or HeLa-MAGI-88C indicate that SI/NSI designation is not meaningful because all viral strains formed syncytia if the correct co-receptor was present. These experiments show that syncytium formation is more likely a marker for the presence of an appropriate co-receptor on the infected cell, rather than an indication of tropism. Infection of the HeLa-MAGI-88C cells with SIV strains reported in the literature to be non-syncytium forming strains, in particular, SIV<sub>MAC</sub>239, SIV<sub>MNE</sub>c18, and SIV<sub>MNE</sub>170, was remarkable because the size of the syncytia induced in the monolayer was much larger than those induced by any other the HIV strains.

### Example 8

Mouse monoclonal antibodies which specifically recognize 88C were prepared. The antibodies were produced by immunizing mice with a peptide corresponding to the amino terminal twenty amino acids of 88C. The peptide was conjugated to Keyhole Limpet Cyanin (KLH) according to the manufacturer's directions (Pierce, Inject maleimide activated KLH), emulsified in complete Freund's adjuvant and injected into five mice. Two additional injections of conjugated peptide in incomplete Freund's adjuvant occurred at three week intervals. Ten days after the final injection, serum from each of the five mice was tested for immunoreactivity with the twenty amino acid peptide by ELISA. In addition, the immunoreactivity of the sera were tested against intact 88C receptor expressed on the surface of 293 cells by fluorescence activated cell sorting (FACS). The mouse with the best anti-



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88C activity was chosen for spleen cell fusion and production of monoclonal antibodies by standard laboratory methods. Five monoclonal cell lines (227K, 227M, 227N, 227P, 227R) were established which produced antibodies that recognized the peptide by ELISA and the 88C protein on 293 cells by FACS.

- 5 Each antibody was shown to react only with 88C-expressing 293 cells, but not with 293 cells expressing the closely related MCP receptor (CCCKR-2). Each antibody was also shown to recognize 88C expressed transiently in COS cells.

- Rabbit polyclonal antibodies were also generated against 88C. Two rabbits were injected with conjugated amino-terminal peptide as described  
10 above. The rabbits were further immunized by four additional injections of the conjugated amino-terminal peptide. Serum from each of the rabbits (2337J and 2470J) was tested by FACS of 293 cells expressing 88C. The sera specifically recognized 88C on the surface of 293 cells.

- The five anti-88C monoclonal antibodies were tested for their  
15 ability to block infection of cells by SIV, the simian immunodeficiency virus closely related to HIV [Lehner, *et al.*, *Nature Medicine*, 2:767 (1996)]. Simian CD4<sup>+</sup> T cells, which are normally susceptible to infection by SIV, were incubated with the SIV<sub>mac</sub>32HJ5 clone in the presence of the anti-88C monoclonal antibody supernatants diluted 1:5. SIV infection was measured  
20 by determining reverse transcriptase (RT) activity on day nine using the RT detection and quantification method (Quan-T-RT assay kit, Amersham, Arlington Heights, IL). Four of the antibodies were able to block SIV infection: antibody 227K blocked by 53 %, 227M by 59 %, 227N by 47 % and 227P by 81 %. Antibody 227R did not block SIV infection.

- 25 The five monoclonal antibodies raised against human 88C amino-terminal peptide were also tested for reactivity against macaque 88C (SEQ ID NO: X) (which has two amino acid differences from human 88C within the amino-terminal peptide region). The coding regions of human 88C and macaque 88C were cloned into the expression vector pcDNA3  
30 (Invitrogen). These expression plasmids were used to transfect COS cells using DEAE. The empty vector was used as a negative control. Three days after transfection, cells were harvested and incubated with the five anti-88C

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monoclonal antibodies and prepared for FACS. The results showed that four of the five antibodies (227K, 227M, 227N, 227P) recognized macaque 88C while one (227R) did not. All five antibodies recognized the transfected human 88C, and none cross-reacted with cells transfected with vector alone.

5

### Example 9

Additional methods may be used to identify ligands and modulators of the chemokine receptors of the invention.

In one embodiment, the invention comprehends a direct assay for ligands. Detectably labeled test compounds are exposed to membrane preparations presenting chemokine receptors in a functional conformation. For example, HEK-293 cells, or tissue culture cells, are transfected with an expression vehicle encoding a chemokine receptor. A membrane preparation is then made from the transfected cells expressing the chemokine receptor. The membrane preparation is exposed to <sup>125</sup>I-labeled test compounds (*e.g.*, chemokines) and incubated under suitable conditions (*e.g.*, 10 minutes at 37°C). The membranes, with any bound test compounds, are then collected on a filter by vacuum filtration and washed to remove unbound test compounds. The radioactivity associated with the bound test compound is then quantitated by subjecting the filters to liquid scintillation spectrophotometry. The specificity of test compound binding may be confirmed by repeating the assay in the presence of increasing quantities of unlabeled test compound and noting the level of competition for binding to the receptor. These binding assays can also identify modulators of chemokine receptor binding. The previously described binding assay may be performed with the following modifications. In addition to detectably labeled test compound, a potential modulator is exposed to the membrane preparation. An increased level of membrane-associated label indicates the potential modulator is an activator; a decreased level of membrane-associated label indicates the potential modulator is an inhibitor of chemokine receptor binding.

30

In another embodiment, the invention comprehends indirect assays for identifying receptor ligands that exploit the coupling of chemokine

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receptors to G proteins. As reviewed in *Linder et al., Sci. Am.*, 267:56-65 (1992), during signal transduction, an activated receptor interacts with a G protein, in turn activating the G protein. The G protein is activated by exchanging GDP for GTP. Subsequent hydrolysis of the G protein-bound GTP deactivates the G protein. One assay for G protein activity therefore monitors the release of  $^{32}\text{P}_i$  from  $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$ . For example, approximately  $5 \times 10^7$  HEK-293 cells harboring plasmids of the invention are grown in MEM + 10% FCS. The growth medium is supplemented with 5 mCi/ml  $[\text{}^{32}\text{P}]\text{-sodium phosphate}$  for 2 hours to uniformly label nucleotide pools. The cells are subsequently washed in a low-phosphate isotonic buffer. One aliquot of washed cells is then exposed to a test compound while a second aliquot of cells is treated similarly, but without exposure to the test compound. Following an incubation period (*e.g.*, 10 minutes), cells are pelleted, lysed and nucleotide compounds fractionated using thin layer chromatography developed with 1 M LiCl. Labeled GTP and GDP are identified by co-developing known standards. The labeled GTP and GDP are then quantitated by autoradiographic techniques that are standard in the art. Relatively high levels of  $^{32}\text{P}$ -labeled GDP identify test compounds as ligands. This type of GTP hydrolysis assay is also useful for the identification of modulators of chemokine receptor binding. The aforementioned assay is performed in the presence of a potential modulator. An intensified signal resulting from a relative increase in GTP hydrolysis, producing  $^{32}\text{P}$ -labeled GDP, indicates a relative increase in receptor activity. The intensified signal therefore identifies the potential modulator as an activator. Conversely, a diminished relative signal for  $^{32}\text{P}$ -labeled GDP, indicative of decreased receptor activity, identifies the potential modulator as an inhibitor of chemokine receptor binding.

The activities of G protein effector molecules (*e.g.*, adenylyl cyclase, phospholipase C, ion channels, and phosphodiesterases) are also amenable to assay. Assays for the activities of these effector molecules have been previously described. For example, adenylyl cyclase, which catalyzes the synthesis of cyclic adenosine monophosphate (cAMP), is activated by G proteins. Therefore, ligand binding to a chemokine receptor that activates a

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G protein, which in turn activates adenylyl cyclase, can be detected by monitoring cAMP levels in a recombinant host cell of the invention. Implementing appropriate controls understood in the art, an elevated level of intracellular cAMP can be attributed to a ligand-induced increase in receptor activity, thereby identifying a ligand. Again using controls understood in the art, a relative reduction in the concentration of cAMP would indirectly identify an inhibitor of receptor activity. The concentration of cAMP can be measured by a commercial enzyme immunoassay. For example, the BioTrak Kit provides reagents for a competitive immunoassay. (Amersham, Inc., Arlington Heights, IL). Using this kit according to the manufacturer's recommendations, a reaction is designed that involves competing unlabeled cAMP with cAMP conjugated to horseradish peroxidase. The unlabeled cAMP may be obtained, for example, from activated cells expressing the chemokine receptors of the invention. The two compounds compete for binding to an immobilized anti-cAMP antibody. After the competition reaction, the immobilized horseradish peroxidase-cAMP conjugate is quantitated by enzyme assay using a tetramethylbenzidine/H<sub>2</sub>O<sub>2</sub> single-pot substrate with detection of colored reaction products occurring at 450 nm. The results provide a basis for calculating the level of unlabeled cAMP, using techniques that are standard in the art. In addition to identifying ligands binding to chemokine receptors, the cAMP assay can also be used to identify modulators of chemokine receptor binding. Using recombinant host cells of the invention, the assay is performed as previously described, with the addition of a potential modulator of chemokine receptor activity. By using controls that are understood in the art, a relative increase or decrease in intracellular cAMP levels reflects the activation or inhibition of adenylyl cyclase activity. The level of adenylyl cyclase activity, in turn, reflects the relative activity of the chemokine receptor of interest. A relatively elevated level of chemokine receptor activity identifies an activator; a relatively reduced level of receptor activity identifies an inhibitor of chemokine receptor activity.

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While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: ICOS Corporation.  
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- (ii) TITLE OF INVENTION: Chemokine Receptor Materials and Methods
- (iii) NUMBER OF SEQUENCES: 20
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  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3383 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 55..1110
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88C polynucleotide and amino acid sequences"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGAAGAGCTG AGACATCCGT TCCCCTACAA GAAACTCTCC CCGGGTGGAA CAAG ATG  
Met  
1

57

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GAT TAT CAA GTG TCA AGT CCA ATC TAT GAC ATC AAT TAT TAT ACA TCG Asp Tyr Gln Val Ser Ser Pro Ile Tyr Asp Ile Asn Tyr Tyr Thr Ser 5 10 15	105
GAG CCC TGC CAA AAA ATC AAT GTG AAG CAA ATC GCA GCC CGC CTC CTG Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg Leu Leu 20 25 30	153
CCT CCG CTC TAC TCA CTG GTG TTC ATC TTT GGT TTT GTG GGC AAC ATG Pro Pro Leu Tyr Ser Leu Val Phe Ile Phe Gly Phe Val Gly Asn Met 35 40 45	201
CTG GTC ATC CTC ATC CTG ATA AAC TGC AAA AGG CTG AAG AGC ATG ACT Leu Val Ile Leu Ile Leu Ile Asn Cys Lys Arg Leu Lys Ser Met Thr 50 55 60 65	249
GAC ATC TAC CTG CTC AAC CTG GCC ATC TCT GAC CTG TTT TTC CTT CTT Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Phe Phe Leu Leu 70 75 80	297
ACT GTC CCC TTC TGG GCT CAC TAT GCT GCC GCC CAG TGG GAC TTT GGA Thr Val Pro Phe Trp Ala His Tyr Ala Ala Ala Gln Trp Asp Phe Gly 85 90 95	345
AAT ACA ATG TGT CAA CTC TTG ACA GGG CTC TAT TTT ATA GGC TTC TTC Asn Thr Met Cys Gln Leu Leu Thr Gly Leu Tyr Phe Ile Gly Phe Phe 100 105 110	393
TCT GGA ATC TTC TTC ATC ATC CTC CTG ACA ATC GAT AGG TAC CTG GCT Ser Gly Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr Leu Ala 115 120 125	441
GTC GTC CAT GCT GTG TTT GCT TTA AAA GCC AGG ACG GTC ACC TTT GGG Val Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe Gly 130 135 140 145	489
GTG GTG ACA AGT GTG ATC ACT TGG GTG GTG GCT GTG TTT GCG TCT CTC Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser Leu 150 155 160	537
CCA GGA ATC ATC TTT ACC AGA TCT CAA AAA GAA GGT CTT CAT TAC ACC Pro Gly Ile Ile Phe Thr Arg Ser Gln Lys Glu Gly Leu His Tyr Thr 165 170 175	585
TGC AGC TCT CAT TTT CCA TAC AGT CAG TAT CAA TTC TGG AAG AAT TTC Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn Phe 180 185 190	633
CAG ACA TTA AAG ATA GTC ATC TTG GGG CTG GTC CTG CCG CTG CTT GTC Gln Thr Leu Lys Ile Val Ile Leu Gly Leu Val Leu Pro Leu Leu Val 195 200 205	681
ATG GTC ATC TGC TAC TCG GGA ATC CTA AAA ACT CTG CTT CGG TGT CGA Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys Arg 210 215 220 225	729
AAT GAG AAG AAG AGG CAC AGG GCT GTG AGG CTT ATC TTC ACC ATC ATG Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile Met 230 235 240	777
ATT GTT TAT TTT CTC TTC TGG GCT CCC TAC AAC ATT GTC CTT CTC CTG Ile Val Tyr Phe Leu Phe Trp Ala Pro Tyr Asn Ile Val Leu Leu Leu 245 250 255	825
AAC ACC TTC CAG GAA TTC TTT GGC CTG AAT AAT TGC AGT AGC TCT AAC Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser Asn 260 265 270	873

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AGG TTG GAC CAA GCT ATG CAG GTG ACA GAG ACT CTT GGG ATG ACG CAC	921
Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr His	
275 280 285	
TGC TGC ATC AAC CCC ATC ATC TAT GCC TTT GTC GGG GAG AAG TTC AGA	969
Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe Arg	
290 295 300 305	
AAC TAC CTC TTA GTC TTC TTC CAA AAG CAC ATT GCC AAA CGC TTC TGC	1017
Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe Cys	
310 315 320	
AAA TGC TGT TCT ATT TTC CAG CAA GAG GCT CCC GAG CGA GCA AGC TCA	1065
Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser Ser	
325 330 335	
GTT TAC ACC CGA TCC ACT GGG GAG CAG GAA ATA TCT GTG GGC TTG	1110
Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu	
340 345 350	
1170	
TGACACGGAC TCAAGTGGGC TGGTGACCCA GTCAGAGTTG TGCACATGGC TTAGTTTTCA	
1230	
TACACAGCCT GGGCTGGGGG TGGGGTGGGA GAGGTCTTTT TAAAAAGGAA GTTACTGTTA	
1290	
TAGAGGGTCT AAGATTCATC CATTTATTTG GCATCTGTTT AAAGTAGATT AGATCTTTTA	
1350	
AGCCCATCAA TTATAGAAAG CCAAATCAAA ATATGTTGAT GAAAAATAGC AACCTTTTTA	
1410	
TCTCCCTTC ACATGCATCA AGTTATTGAC AAACCTCTCC TCACTCCGA AAGTTCCTTA	
1470	
TGTATATTTA AAAGAAAGCC TCAGAGAATT GCTGATTCTT GAGTTTAGTG ATCTGAACAG	
1530	
AAATACCAAA ATTATTTTCTG AAATGTACAA CTTTTTACCT AGTACAAGGC AACATATAGG	
1590	
TTGTAAATGT GTTTAAACA GGTCTTTGTC TTGCTATGGG GAGAAAAGAC ATGAATATGA	
1650	
TTAGTAAAGA AATGACACTT TTCATGTGTG ATTTCCCCTC CAAGGTATGG TTAATAAGTT	
1710	
TCACTGACTT AGAACCAGGC GAGAGACTTG TGGCCTGGGA GAGCTGGGGA AGCTTCTTAA	
1770	
ATGAGAAGGA ATTTGAGTTG GATCATCTAT TGCTGGCAA GACAGAAGCC TCACTGCAAG	
1830	
CACTGCATGG GCAAGCTTGG CTGTAGAAGG AGACAGAGCT GGTGGGAAG ACATGGGGAG	
1890	
GAAGGACAAG GCTAGATCAT GAAGAACCTT GACGGCATTG CTCCGTCTAA GTCATGAGCT	
1950	
GAGCAGGGAG ATCCTGGTTG GTGTTGCAGA AGGTTTACTC TGTGGCCAAA GGAGGGTCAG	
2010	
GAAGGATGAG CATTTAGGGC AAGGAGACCA CCAACAGCCC TCAGGTCAGG GTGAGGATGG	
2070	
CCTCTGCTAA GCTCAAGGCG TGAGGATGGG AAGGAGGGAG GTATTCGTAA GGATGGGAAG	
2130	
GAGGGAGGTA TTCGTGCAGC ATATGAGGAT GCAGAGTCAG CAGAACTGGG GTGGATTTGG	
2190	
TTTGGAAGTG AGGGTCAGAG AGGAGTCAGA GAGAATCCCT AGTCTTCAAG CAGATTGGAG	
2250	
AAACCTTGA AAAGACATCA AGCACAGAAG GAGGAGGAGG AGGTTTAGGT CAAGAAGAAG	
2310	
ATGGATTGGT GTAAAAGGAT GGGTCTGGTT TGCAGAGCTT GAACACAGTC TCACCCAGAC	
2370	
TCCAGGCTGT CTTTCACTGA ATGCTTCTGA CTTCATAGAT TTCCTTCCCA TCCCAGCTGA	
2430	
AATACTGAGG GGTCTCCAGG AGGAGACTAG ATTTATGAAT ACACGAGGTA TGAGGTCTAG	
2490	
GAACATACTT CAGCTCACAC ATGAGATCTA GGTGAGGATT GATTACCTAG TAGTCATTTT	
2550	
ATGGGTTGTT GGGAGGATTC TATGAGGCAA CCACAGGCAG CATTTAGCAC ATACTACACA	



(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 352 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEO ID NO:2:

Met	Asp	Tyr	Gln	Val	Ser	Ser	Pro	Ile	Tyr	Asp	Ile	Asn	Tyr	Thr	
1				5					10					15	
Ser	Glu	Pro	Cys	Gln	Lys	Ile	Asn	Val	Lys	Gln	Ile	Ala	Ala	Arg	Leu
			20					25					30		
Leu	Pro	Pro	Leu	Tyr	Ser	Leu	Val	Phe	Ile	Phe	Gly	Phe	Val	Gly	Asn
		35					40					45			
Met	Leu	Val	Ile	Leu	Ile	Leu	Ile	Asn	Cys	Lys	Arg	Leu	Lys	Ser	Met
	50					55					60				
Thr	Asp	Ile	Tyr	Leu	Leu	Asn	Leu	Ala	Ile	Ser	Asp	Leu	Phe	Phe	Leu
65					70					75					80
Leu	Thr	Val	Pro	Phe	Trp	Ala	His	Tyr	Ala	Ala	Ala	Gln	Trp	Asp	Phe
				85					90					95	
Gly	Asn	Thr	Met	Cys	Gln	Leu	Leu	Thr	Gly	Leu	Tyr	Phe	Ile	Gly	Phe
			100					105					110		
Phe	Ser	Gly	Ile	Phe	Phe	Ile	Ile	Leu	Leu	Thr	Ile	Asp	Arg	Tyr	Leu
		115					120					125			

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Ala Val Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe  
 130 135 140

Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser  
 145 150 155 160

Leu Pro Gly Ile Ile Phe Thr Arg Ser Gln Lys Glu Gly Leu His Tyr  
 165 170 175

Thr Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn  
 180 185 190

Phe Gln Thr Leu Lys Ile Val Ile Leu Gly Leu Val Leu Pro Leu Leu  
 195 200 205

Val Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys  
 210 215 220

Arg Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile  
 225 230 235 240

Met Ile Val Tyr Phe Leu Phe Trp Ala Pro Tyr Asn Ile Val Leu Leu  
 245 250 255

Leu Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser  
 260 265 270

Asn Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr  
 275 280 285

His Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe  
 290 295 300

Arg Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe  
 305 310 315 320

Cys Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser  
 325 330 335

Ser Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu  
 340 345 350

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1915 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 362..1426

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B polynucleotide and amino acid sequences"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATAATAATGA TTATTATATT GTTATCATT A TCTAGCCTGT TTTTTCCTGT TTTGTATTTC 60

TTCTTTTAA TGCTTTCAGA AATCTGTATC CCCATTCTTC ACCACCACCC CACAACATT 120

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CTGCTTCTTT TCCCATGCCG GGTCACTGCTA ACTTTGAAAG CTTTCTCTCT TTCCTTCCTC	180
AATCCTTTTC CTGGCACCTC TGATATGCCT TTTGAAATTC ATGTTAAAGA ATCCCTAGGC	240
TGCTATCACA TGTGGCATCT TTGTTGAGTA CATGAATAAA TCAACTGGTG TGTTTTACGA	300
AGGATGATTA TGCTTCATTG TGGGATTGTA TTTTCTTCT TCTATCACAG GGAGAAGTGA	360
A ATG ACA ACC TCA CTA GAT ACA GTT GAG ACC TTT GGT ACC ACA TCC	406
Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser	
1 5 10 15	
TAC TAT GAT GAC GTG GGC CTG CTC TGT GAA AAA GCT GAT ACC AGA GCA	454
Tyr Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala	
20 25 30	
CTG ATG GCC CAG TTT GTG CCC CCG CTG TAC TCC CTG GTG TTC ACT GTG	502
Leu Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val	
35 40 45	
GGC CTC TTG GGC AAT GTG GTG GTG GTG ATG ATC CTC ATA AAA TAC AGG	550
Gly Leu Leu Gly Asn Val Val Val Val Met Ile Leu Ile Lys Tyr Arg	
50 55 60	
AGG CTC CGA ATT ATG ACC AAC ATC TAC CTG CTC AAC CTG GCC ATT TCG	598
Arg Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser	
65 70 75	
GAC CTG CTC TTC CTC GTC ACC CTT CCA TTC TGG ATC CAC TAT GTC AGG	646
Asp Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg	
80 85 90 95	
GGG CAT AAC TGG GTT TTT GGC CAT GGC ATG TGT AAG CTC CTC TCA GGG	694
Gly His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly	
100 105 110	
TTT TAT CAC ACA GGC TTG TAC AGC GAG ATC TTT TTC ATA ATC CTG CTG	742
Phe Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu	
115 120 125	
ACA ATC GAC AGG TAC CTG GCC ATT GTC CAT GCT GTG TTT GCC CTT CGA	790
Thr Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg	
130 135 140	
GCC CGG ACT GTC ACT TTT GGT GTC ATC ACC AGC ATC GTC ACC TGG GGC	838
Ala Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly	
145 150 155	
CTG GCA GTG CTA GCA GCT CTT CCT GAA TTT ATC TTC TAT GAG ACT GAA	886
Leu Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu	
160 165 170 175	
GAG TTG TTT GAA GAG ACT CTT TGC AGT GCT CTT TAC CCA GAG GAT ACA	934
Glu Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr	
180 185 190	
GTA TAT AGC TGG AGG CAT TTC CAC ACT CTG AGA ATG ACC ATC TTC TGT	982
Val Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys	
195 200 205	
CTC GTT CTC CCT CTG CTC GTT ATG GCC ATC TGC TAC ACA GGA ATC ATC	1030
Leu Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile	
210 215 220	
AAA ACG CTG CTG AGG TGC CCC AGT AAA AAA AAG TAC AAG GCC ATC CGG	1078
Lys Thr Leu Leu Arg Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg	
225 230 235	

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CTC	ATT	TTT	GTC	ATC	ATG	GCG	GTG	TTT	TTC	ATT	TTC	TGG	ACA	CCC	TAC	1126												
Leu	Ile	Phe	Val	Ile	Met	Ala	Val	Phe	Phe	Ile	Phe	Trp	Thr	Pro	Tyr													
240					245					250					255													
AAT	GTG	GCT	ATC	CTT	CTC	TCT	TCC	TAT	CAA	TCC	ATC	TTA	TTT	GGA	AAT	1174												
Asn	Val	Ala	Ile	Leu	Leu	Ser	Ser	Tyr	Gln	Ser	Ile	Leu	Phe	Gly	Asn													
				260					265					270														
GAC	TGT	GAG	CGG	AGC	AAG	CAT	CTG	GAC	CTG	GTC	ATG	CTG	GTG	ACA	GAG	1222												
Asp	Cys	Glu	Arg	Ser	Lys	His	Leu	Asp	Leu	Val	Met	Leu	Val	Thr	Glu													
			275					280					285															
GTG	ATC	GCC	TAC	TCC	CAC	TGC	TGC	ATG	AAC	CCG	GTG	ATC	TAC	GCC	TTT	1270												
Val	Ile	Ala	Tyr	Ser	His	Cys	Cys	Met	Asn	Pro	Val	Ile	Tyr	Ala	Phe													
		290					295					300																
GTT	GGA	GAG	AGG	TTC	CGG	AAG	TAC	CTG	CGC	CAC	TTC	TTC	CAC	AGG	CAC	1318												
Val	Gly	Glu	Arg	Phe	Arg	Lys	Tyr	Leu	Arg	His	Phe	Phe	His	Arg	His													
	305					310					315																	
TTG	CTC	ATG	CAC	CTG	GGC	AGA	TAC	ATC	CCA	TTC	CTT	CCT	AGT	GAG	AAG	1366												
Leu	Leu	Met	His	Leu	Gly	Arg	Tyr	Ile	Pro	Phe	Leu	Pro	Ser	Glu	Lys													
320					325					330					335													
CTG	GAA	AGA	ACC	AGC	TCT	GTC	TCT	CCA	TCC	ACA	GCA	GAG	CCG	GAA	CTC	1414												
Leu	Glu	Arg	Thr	Ser	Ser	Val	Ser	Pro	Ser	Thr	Ala	Glu	Pro	Glu	Leu													
				340					345					350														
TCT	ATT	GTG	TTT	TAGGTCAGAT GCAGAAAATT GCCTAAAGAG GAAGGACCAA												1466												
Ser	Ile	Val	Phe																									
			355																									
GGAGATGAAG CAAACACATT AAGCCTTCCA CACTCACCTC TAAAACAGTC CTTCAAACCTT																1526												
CCAGTGCAAC ACTGAAGCTC TTGAAGACAC TGAAATATAC ACACAGCAGT AGCAGTAGAT																1586												
GCATGTACCC TAAGGTCATT ACCACAGGCC AGGGGCTGGG CAGCGTACTC ATCATCAACC																1646												
CTAAAAGCA GAGCTTTGCT TCTCTCTCTA AAATGAGTTA CCTACATTTT AATGCACCTG																1706												
AATGTTAGAT AGTTACTATA TGCCGCTACA AAAAGGTAAA ACTTTTTATA TTTTATACAT																1766												
TAACTTCAGC CAGCTATTGA TATAAATAAA ACATTTTCAC ACAATACAAT AAGTTAACTA																1826												
TTTTATTTTC TAATGTGCCT AGTTCTTTCC CTGCTTAATG AAAAGCTTGT TTTTTCAGTG																1886												
TGAATAAATA ATCGTAAGCA ACAAAAAAA																1915												

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 355 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B amino acid sequence"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Thr	Thr	Ser	Leu	Asp	Thr	Val	Glu	Thr	Phe	Gly	Thr	Thr	Ser	Tyr
1				5				10						15	

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Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu  
                     20                    25                    30  
 Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val Gly  
                     35                    40                    45  
 Leu Leu Gly Asn Val Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg  
                     50                    55                    60  
 Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp  
                     65                    70                    75                    80  
 Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly  
                     85                    90                    95  
 His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe  
                     100                    105  
 Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr  
                     115                    120                    125  
 Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala  
                     130                    135                    140  
 Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu  
                     145                    150                    155                    160  
 Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Glu  
                     165                    170                    175  
 Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr Val  
                     180                    185                    190  
 Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys Leu  
                     195                    200                    205  
 Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile Lys  
                     210                    215                    220  
 Thr Leu Leu Arg Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg Leu  
                     225                    230                    235                    240  
 Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn  
                     245                    250                    255  
 Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn Asp  
                     260                    265                    270  
 Cys Glu Arg Ser Lys His Leu Asp Leu Val Met Leu Val Thr Glu Val  
                     275                    280                    285  
 Ile Ala Tyr Ser His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe Val  
                     290                    295                    300  
 Gly Glu Arg Phe Arg Lys Tyr Leu Arg His Phe Phe His Arg His Leu  
                     305                    310                    315                    320  
 Leu Met His Leu Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys Leu  
                     325                    330                    335  
 Glu Arg Thr Ser Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu Ser  
                     340                    345                    350  
 Ile Val Phe  
                     355

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## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "V28degf2"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACGGATCCA TYGAYAGRTA CCTGGCYATY GTCC

34

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "V28degr2"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTAAGCTTT TRTAGGGDGT CCAYAAGAGY AA

32

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88c-r4"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATAAGCCTC ACAGCCCTGT G

21

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88c-rlb"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:  
GCTAAGCTTG ATGACTATCT TTAATGTC 28

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:  
(A) NAME/KEY: misc\_feature  
(D) OTHER INFORMATION: /= "88-2B-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
CCCTCTAGAC TAAACACAA TAGAGAG 27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:  
(A) NAME/KEY: misc\_feature  
(D) OTHER INFORMATION: /= "88-2B-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
GCTAAGCTTA TCACAGGGAG AAGTGAAATG 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:  
(A) NAME/KEY: misc\_feature  
(D) OTHER INFORMATION: /= "88-2B-f1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:  
AGTGCTAGCA GCTCTTCCTG 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88-2B-r1"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCAGCGTT TTGATGATTC

20

- (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA

- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88C-f1"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTGTTTGCT TTAAAAGCC

19

- (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA

- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "88C-r3"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAAGCCTCAC AGCCCTG

17

- (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA

- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (D) OTHER INFORMATION: /= "CCCKR1(2)-5' Primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGTAAGCTTA GAGAAGCCGG GATGGGAA

28

- (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid



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(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc\_feature  
(D) OTHER INFORMATION: /= "CCCKR-3' Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCTCTAGAG TCAGAGACCA GCAGA

25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GACAAGCTTC ACAGGGTGA ACAAGATG

28

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCTCTAGAC CACTTGAGTC CGTGTC

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1059 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..1056

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

- 56 -

ATG Met 1	GAC Asp	TAT Tyr	CAA Gln	GTG Val 5	TCA Ser	AGT Ser	CCA Pro	ACC Thr	TAT Tyr 10	GAC Asp	ATC Ile	GAT Asp	TAT Tyr 15	TAT Tyr	ACA Thr	48
TCG Ser	GAA Glu	CCC Pro	TGC Cys 20	CAA Gln	AAA Lys	ATC Ile	AAT Asn	GTG Val 25	AAA Lys	CAA Gln	ATC Ile	GCA Ala	GCC Ala 30	CGC Arg	CTC Leu	96
CTG Leu	CCT Pro	CCG Pro 35	CTC Leu	TAC Tyr	TCA Ser	CTG Leu	GTG Val 40	TTC Phe	ATC Ile	TTT Phe	GGT Gly	TTT Phe 45	GTG Val	GGC Gly	AAC Asn	144
ATA Ile 50	CTG Leu	GTC Val	GTC Val	CTC Leu	ATC Ile	CTG Leu 55	ATA Ile	AAC Asn	TGC Cys	AAA Lys	AGG Arg 60	CTG Leu	AAA Lys	AGC Ser	ATG Met	192
ACT Thr 65	GAC Asp	ATC Ile	TAC Tyr	CTG Leu	CTC Leu 70	AAC Asn	CTG Leu	GCC Ala	ATC Ile	TCT Ser 75	GAC Asp	CTG Leu	CTT Leu	TTC Phe	CTT Leu 80	240
CTT Leu	ACT Thr	GTC Val	CCC Pro	TTC Phe 85	TGG Trp	GCT Ala	CAC His	TAT Tyr 90	GCT Ala	GCT Ala	GCC Ala	CAG Gln	TGG Trp	GAC Asp 95	TTT Phe	288
GGA Gly	AAT Asn	ACA Thr	ATG Met 100	TGT Cys	CAA Gln	CTC Leu	TTG Leu	ACA Thr 105	GGG Gly	CTC Leu	TAT Tyr	TTT Phe 110	ATA Ile	GGC Gly	TTC Phe	336
TTC Phe	TCT Ser	GGA Gly 115	ATC Ile	TTC Phe	TTC Phe	ATC Ile	ATC Ile 120	CTC Leu	CTG Leu	ACA Thr	ATC Ile	GAT Asp 125	AGG Arg	TAC Tyr	CTG Leu	384
GCT Ala 130	ATC Ile	GTC Val	CAT His	GCT Ala	GTG Val	TTT Phe 135	GCT Ala	TTA Leu	AAA Lys	GCC Ala	AGG Arg 140	ACA Thr	GTC Val	ACC Thr	TTT Phe	432
GGG Gly 145	GTG Val	GTG Val	ACA Thr	AGT Ser	GTG Val 150	ATC Ile	ACT Thr	TGG Trp	GTG Val	GTG Val 155	GCT Ala	GTG Val	TTT Phe	GCC Ala	TCT Ser 160	480
CTC Leu	CCA Pro	GGA Gly	ATC Ile	ATC Ile	TTT Phe 165	ACC Thr	AGA Arg	TCT Ser	CAG Gln	AGA Arg	GAA Glu	GGT Gly	CTT Leu	CAT His 175	TAC Tyr	528
ACC Thr	TGC Cys	AGC Ser	TCT Ser 180	CAT His	TTT Phe	CCA Pro	TAC Tyr	AGT Ser 185	CAG Gln	TAT Tyr	CAA Gln	TTC Phe	TGG Trp 190	AAG Lys	AAT Asn	576
TTT Phe	CAG Gln	ACA Thr 195	TTA Leu	AAG Lys	ATG Met	GTC Val 200	ATC Ile	TTG Leu	GGG Gly	CTG Leu	GTC Val	CTG Leu 205	CCG Pro	CTG Leu	CTT Leu	624
GTC Val 210	ATG Met	GTC Val	ATC Ile	TGC Cys	TAC Tyr	TCG Ser 215	GGA Gly	ATC Ile	CTG Leu	AAA Lys	ACT Thr 220	CTG Leu	CTT Leu	CGG Arg	TGT Cys	672
CGA Arg 225	AAC Asn	GAG Glu	AAG Lys	AAG Lys	AGG Arg 230	CAC His	AGG Arg	GCT Ala	GTG Val	AGG Arg 235	CTT Leu	ATC Ile	TTC Phe	ACC Thr	ATC Ile 240	720
ATG Met	ATT Ile	GTT Val	TAT Tyr	TTT Phe 245	CTC Leu	TTG Leu	TGG Trp	GCT Ala	CCC Pro 250	TAC Tyr	AAC Asn	ATT Ile	GTC Val	CTT Leu 255	CTC Leu	768
CTG Leu	AAC Asn	ACC Thr	TTC Phe 260	CAG Gln	GAA Glu	TTC Phe	TTT Phe	GGC Gly 265	CTG Leu	AAT Asn	AAT Asn	TGC Cys	AGT Ser	AGC Ser	TCT Ser	816

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AAC AGG TTG GAC CAA GCC ATG CAG GTG ACA GAG ACT CTT GGG ATG ACA	864
Asn Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr	
275 280 285	
CAC TGC TGC ATC AAC CCC ATC ATC TAT GCC TTT GTC GGG GAG AAG TTC	912
His Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe	
290 295 300	
AGA AAC TAC CTC TTA GTC TTC TTC CAA AAG CAC ATT GCC AAA CGC TTC	960
Arg Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe	
305 310 315 320	
TGC AAA TGC TGT TCC ATT TTC CAG CAA GAG GCT CCC GAG CGA GCA AGT	1008
Cys Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser	
325 330 335	
TCA GTT TAC ACC CGA TCC ACT GGG GAG CAG GAA ATA TCT GTG GGC TTG	1056
Ser Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu	
340 345 350	
TGA	1059

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Met Asp Tyr Gln Val Ser Ser Pro Thr Tyr Asp Ile Asp Tyr Tyr Thr	
1 5 10 15	
Ser Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg Leu	
20 25 30	
Leu Pro Pro Leu Tyr Ser Leu Val Phe Ile Phe Gly Phe Val Gly Asn	
35 40 45	
Ile Leu Val Val Leu Ile Leu Ile Asn Cys Lys Arg Leu Lys Ser Met	
50 55 60	
Thr Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Leu	
65 70 75 80	
Leu Thr Val Pro Phe Trp Ala His Tyr Ala Ala Ala Gln Trp Asp Phe	
85 90 95	
Gly Asn Thr Met Cys Gln Leu Leu Thr Gly Leu Tyr Phe Ile Gly Phe	
100 105 110	
Phe Ser Gly Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr Leu	
115 120 125	
Ala Ile Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe	
130 135 140	
Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser	
145 150 155 160	
Leu Pro Gly Ile Ile Phe Thr Arg Ser Gln Arg Glu Gly Leu His Tyr	
165 170 175	

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Thr	Cys	Ser	Ser	His	Phe	Pro	Tyr	Ser	Gln	Tyr	Gln	Phe	Trp	Lys	Asn
			180					185					190		
Phe	Gln	Thr	Leu	Lys	Met	Val	Ile	Leu	Gly	Leu	Val	Leu	Pro	Leu	Leu
		195					200					205			
Val	Met	Val	Ile	Cys	Tyr	Ser	Gly	Ile	Leu	Lys	Thr	Leu	Leu	Arg	Cys
	210					215					220				
Arg	Asn	Glu	Lys	Lys	Arg	His	Arg	Ala	Val	Arg	Leu	Ile	Phe	Thr	Ile
225					230					235					240
Met	Ile	Val	Tyr	Phe	Leu	Leu	Trp	Ala	Pro	Tyr	Asn	Ile	Val	Leu	Leu
				245					250					255	
Leu	Asn	Thr	Phe	Gln	Glu	Phe	Phe	Gly	Leu	Asn	Asn	Cys	Ser	Ser	Ser
			260					265					270		
Asn	Arg	Leu	Asp	Gln	Ala	Met	Gln	Val	Thr	Glu	Thr	Leu	Gly	Met	Thr
		275					280					285			
His	Cys	Cys	Ile	Asn	Pro	Ile	Ile	Tyr	Ala	Phe	Val	Gly	Glu	Lys	Phe
	290					295					300				
Arg	Asn	Tyr	Leu	Leu	Val	Phe	Phe	Gln	Lys	His	Ile	Ala	Lys	Arg	Phe
305					310					315					320
Cys	Lys	Cys	Cys	Ser	Ile	Phe	Gln	Gln	Glu	Ala	Pro	Glu	Arg	Ala	Ser
				325					330					335	
Ser	Val	Tyr	Thr	Arg	Ser	Thr	Gly	Glu	Gln	Glu	Ile	Ser	Val	Gly	Leu
			340					345					350		

**CLAIMS**

We claim:

1. A purified and isolated polynucleotide encoding the amino acid sequence of chemokine receptor 88-2B set out in SEQ ID NO:4.
2. A polynucleotide according to claim 1 wherein the polynucleotide is DNA.
3. A polynucleotide according to claim 2 wherein the polynucleotide is genomic DNA.
4. A polynucleotide according to claim 2 wherein the polynucleotide is cDNA.
5. A polynucleotide according to claim 1 which is a wholly or partially chemically synthesized DNA.
6. An RNA transcript of the polynucleotide of claim 2.
7. A cDNA according to claim 4 comprising the DNA of SEQ ID NO:3.
8. A biologically functional DNA vector comprising a DNA according to claim 2.
9. A vector according to claim 8 wherein said DNA is operatively linked to a DNA expression control sequence.
10. A host cell stably transformed or transfected with a DNA according to claim 1 in a manner allowing expression of said DNA.

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11. A method for producing an 88-2B polypeptide comprising the steps of growing a host cell according to claim 10 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.

12. A polynucleotide encoding an 88-2B polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 3.

13. A purified and isolated polypeptide comprising the chemokine receptor 88-2B amino acid sequence set out in SEQ ID NO:4.

14. An antibody product that specifically binds a polypeptide comprising the 88-2B amino acid sequence set out in SEQ ID NO:4.

15. A hybridoma producing an antibody product according to claim 14.

16. A purified and isolated polynucleotide encoding the amino acid sequence of chemokine receptor 88C set out in SEQ ID NO:2.

17. A polynucleotide according to claim 16 wherein the polynucleotide is DNA.

18. A polynucleotide according to claim 17 wherein the polynucleotide is genomic DNA.

19. A polynucleotide according to claim 17 wherein the polynucleotide is a cDNA.

20. A polynucleotide according to claim 16 which is a wholly or partially chemically synthesized DNA.

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21. An RNA transcript of the polynucleotide of claim 17.
22. A cDNA according to claim 19 comprising the DNA of SEQ ID NO:1.
23. A biologically functional DNA vector comprising a DNA according to claim 17.
24. A vector according to claim 23 wherein said DNA is operatively linked to a DNA expression control sequence.
25. A host cell stably transformed or transfected with a DNA according to claim 16 in a manner allowing expression of said DNA.
26. A method for producing an 88C polypeptide comprising the steps of growing a host cell according to claim 25 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.
27. A polynucleotide encoding an 88C polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 1.
28. A purified and isolated polypeptide comprising the chemokine receptor 88C amino acid sequence set out in SEQ ID NO:2.
29. An antibody product that specifically binds a polypeptide comprising the 88C amino acid sequence set out in SEQ ID NO:2.
30. A hybridoma producing an antibody product according to claim 29.
31. Hybridoma cell line 227K.

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32. Hybridoma cell line 227M.
33. Hybridoma cell line 227N.
34. Hybridoma cell line 227P.
35. Hybridoma cell line 227R.
36. A purified and isolated polynucleotide encoding the amino acid sequence of macaque chemokine receptor 88C set out in SEQ ID NO: 20.
37. A polynucleotide according to claim 36 wherein the polynucleotide is DNA.
38. A polynucleotide according to claim 37 wherein the polynucleotide is genomic DNA.
39. A polynucleotide according to claim 37 wherein the polynucleotide is a cDNA.
40. A polynucleotide according to claim 36 which is a wholly or partially chemically synthesized DNA.
41. An RNA transcript of the polynucleotide of claim 37.
42. A cDNA according to claim 39 comprising the DNA of SEQ ID NO:1.
43. A biologically functional DNA vector comprising a DNA according to claim 37.



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44. A vector according to claim 43 wherein said DNA is operatively linked to a DNA expression control sequence.

45. A host cell stably transformed or transfected with a DNA according to claim 36 in a manner allowing expression of said DNA.

46. A method for producing a macaque 88C polypeptide comprising the steps of growing a host cell according to claim 45 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.

47. A polynucleotide encoding an 88C polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 19.

48. A purified and isolated polypeptide comprising the macaque chemokine receptor 88C amino acid sequence set out in SEQ ID NO:20.

49. An antibody product that specifically binds a polypeptide comprising the 88C amino acid sequence set out in SEQ ID NO:20.

50. A hybridoma producing an antibody product according to claim 49.

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, C07K 14/705, 16/28, C12N 5/20</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/22698</b> <b>(43) International Publication Date:</b> 26 June 1997 (26.06.97)
<b>(21) International Application Number:</b> PCT/US96/20759 <b>(22) International Filing Date:</b> 20 December 1996 (20.12.96)  <b>(30) Priority Data:</b> 08/575,967 20 December 1995 (20.12.95) US 08/661,393 7 June 1996 (07.06.96) US  <b>(71) Applicant:</b> ICOS CORPORATION [US/US]; 22021 20th Avenue S.E., Bothell, WA 98021 (US).  <b>(72) Inventors:</b> GRAY, Patrick, W.; 1600 40th Avenue, Seattle, WA 98122 (US). SCHWEICKART, Vicki, L.; 1421 Orange Place North, Seattle, WA 98109 (US). RAPORT, Carol, J.; 2300 211th Street, S.E., Bothell, WA 98021 (US).  <b>(74) Agent:</b> BORUN, Michael, F.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606-6402 (US).		<b>(81) Designated States:</b> AU, BR, CA, CN, CZ, FI, HU, JP, MX, NO, PL, RU, SK, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 12 September 1997 (12.09.97)
<b>(54) Title:</b> CHEMOKINE RECEPTORS 88-2B[CKR-3] AND 88C AND THEIR ANTIBODIES  <b>(57) Abstract</b>  The present invention provides polynucleotides that encode the chemokine receptors 88-2B or 88C and materials and methods for the recombinant production of these two chemokine receptors. Also provided are assays utilizing the polynucleotides which facilitate the identification of ligands and modulators of the chemokine receptors. Receptor fragments, ligands, modulators, and antibodies are useful in the detection and treatment of disease states associated with the chemokine receptors such as atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infection, AIDS, and other inflammatory conditions.		

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GA	Gabon			VN	Viet Nam

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/20759

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/12 C07K14/705 C07K16/28 C12N5/20

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, 14 July 1995, MD US, pages 16491-16494, XP002029908 COMBADIÈRE, C. ET AL. : "Cloning and functional expression of a human eosinophil CC Chemokine Receptor" see the whole document ---	1-15
P,X	WO 96 22371 A (LEUKOSITE INC ;BRIGHAM & WOMENS HOSPITAL (US); CHILDRENS MEDICAL C) 25 July 1996 see claims 1-29,38,39 ---	1-15
P,X	WO 96 39437 A (HUMAN GENOME SCIENCES INC ;LI YI (US); RUBEN STEVEN M (US)) 12 December 1996 see page 33, line 7 - page 34, line 11; claim 1 --- -/--	16-50

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

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- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

15 July 1997

Date of mailing of the international search report

22.07.97

Name and mailing address of the ISA

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Fax: (+ 31-70) 340-3016

Authorized officer

Nauche, S

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/20759

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMISTRY, MAR 19 1996, 35 (11) P3362-7, UNITED STATES, XP002035224 SAMSON M ET AL: "Molecular cloning and functional expression of a new human CC-chemokine receptor gene." see the whole document ---	16-50
P,X	NATURE, JUN 20 1996, 381 (6584) P667-73, ENGLAND, XP002035225 DRAGIC T ET AL: "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5 [see comments]" see the whole document ---	16-50
P,X	NATURE, JUN 20 1996, 381 (6584) P661-6, ENGLAND, XP002035226 DENG H ET AL: "Identification of a major co-receptor for primary isolates of HIV-1 [see comments]" see the whole document -----	16-50

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 20759

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 20759

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. Claims: 1-15 A chemokine receptor 88-2B [ CKR-3 chemokine receptor], sequence encoding said receptor. Expression vector and recombinant host cells for the production of chemokine receptor 88-2B [ CKR-3 chemokine receptor]. Antibodies immunoreactive with chemokine receptor 88-2B [ CKR-3 chemokine receptor]. Hybridoma producing said antibodies.
2. Claims: 16-50 chemokine receptor 88-C, sequence encoding said receptor. Expression vector and recombinant host cells for the production of chemokine receptor 88-C. Antibodies immunoreactive with chemokine receptor 88-C. Hybridoma producing said antibodies

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/20759

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9622371 A	25-07-96	AU 5020296 A	07-08-96
WO 9639437 A	12-12-96	AU 2663295 A	24-12-96